Gratuitous supplement

Like many other situations in my life, I found myself in Leuven in September 2008 without even knowing how I got here! I vividly remember that before finishing my master's studies, I thought a lot about what could be the best decision; to continue my PhD studies in Iran or embark on a PhD project abroad. Eventually, I decided to do a PhD abroad. Was it the right decision? Perhaps! Well, I can never know how things would have turned out if I had chosen another path in my life. But did I like it? No doubts, Yes!

As always, I am indebted to many people.

I would like to thank the committee members, Professor Octave, Professor Baes, Professor Baekelandt (it was really sad to hear about your loss as we were preparing the thesis), and Professor Jossin, all of whom kindly accepted to read my thesis and send me very helpful suggestions.

Bart, it has been a great pleasure to work in your lab. Our interaction changed my views about science and I learned many different things from you. The one I like most is this: "first, I should be able to convince myself, and then I might have a chance to convince others as well." Among the various skills I acquired during my PhD studies, the one I appreciate most is "critical thinking". Sometimes, I assumed that because I understood "one", I could also understand "two", as one and one make two! Then, you reminded me that I must understand "and". Thank you for giving me the opportunity to work in such an amazing environment. In your lab, I enjoyed doing science as much as I had fun interacting with people from all around the world. It has been such a remarkable experience that I am almost sorry that I am finishing my PhD!

Bassem, I like to thank you for inviting me to Leuven for the interview five years ago and linking me to Bart. I learned from you that one should have at least one theory, preferably several, for every possible observation. I can just say that I am happy that I got to know a special Drosophila neurobiologist who has a picture of Che in his office and write about Middle East politics (I really think this is unique).Thanks a lot.

I could have never finished this work if I was not lucky enough to meet Carlos and Annette. Carlos, your constructive feedbacks and your positive attitude in difficult

times was a great help. Your never ending smiles convinced me that the “happy scientist” is not just a myth. Annette, It was you who showed me how to combine planning and flexibility (and I guess a bit of diplomacy as well!) to succeed. I know that I was difficult to handle at times but you skillfully showed me what the best way to continue was. It was a great pleasure working with you and thanks for everything.

The first week of the PhD was one of the most confusing weeks in my life because of the new social and scientific surroundings[1]. Luckily, it did not take too long to meet Bruno. Bruno, you helped me to find my way around in the lab and become independent. I remember how quickly you showed me where and when different departmental seminars were, in particular the ones with free sandwiches! That was very useful. You are an amazing friend and I hope we get to travel to Iran together.

I have met so many nice friends and colleagues in the lab that it is actually difficult to have a concise acknowledgment! So expect a long list (stochastic ordering of names!). Lujia (it is never too late to open the grocery), Hugo (there are those letters that are neither written, nor pronounced), Pierre (don’t worry, many roads to Monaco), Tilmann (I consider myself lucky to know you), Carlo (I am sure you will publish nice papers in my journal), Sam (I will thank you in the supplementary acknowledgment), Iryna (Bonjour mon amie!), Smaragda (nice to finish together and you saved my thesis), Anna, Fabrizio, Wim, Zeljko (no comment!), Alexandra, Tomasz, Ellen, Soraia, Liesbeth, Hermien (how is my good friend?), Amaia, Ina, Lucia, Francesc (for the unforgettable western blot of my life), Manuel (for lending me your "boos" card), Gerard (for trying to understand my jokes), Evgenia (really time to say “bye guys”), Samer (with you I realized I sometimes need to replace "why" with "why not"), Dominik, Nobuto (remember the analogy between Kebab and gamma secretase), Vanessa (do not forget, LIFE is like a watermelon!), Marco (the genius of all the time), Hilde (I could not even make it to Belgium without you), Eliane (thanks for helping me with the thesis), Isabel (for the battery of the laptop!), Sabine, Manasi (for the inspiring Ladoo movie), Sulieman (cheers man!), Katrien and Katleen (you knew almost everything), Gert, Zhiyong, Elke (we had a few in the lab, all very nice people), Tine (it was fun working with you), Veronique and Jonas (no PhD for me without you) and Lutgarde (one of my best friends!). Thank you all so

[1] . As I was preparing for the public defense, I realized that the last weeks can be as confusing as the first weeks.

much. I almost forget to thank Pietro, Nadav, Yunhong and Sarah with whom I shared not only an office but also opinions (and gossip!). Amantha, not many people think I can be a good scientist and you are one of them. Thank you so much for helping me to continue in science, not to mention the biscuits! Eventually, a special mention in my thesis should go to my “alarm clock”, without whom I may never have made it!

I would also like to thank the early morning people of the department who prepared everything before we arrive in the lab. Many of them I do not know by name but I truly appreciate their great help.

I would like to take this opportunity to thank the Iranian community of Leuven without whom I would have finished this work a couple of months earlier! In particular, Hamid o Nahid, Hassan o Tina, Omid o Elham and Ahmad o Maryam. Hamid, you rely too much on your brain, it is one of the most overrated organs! Hassan, I should be more respectful to you as a potential future colleague. Omid, I think everyone deserves some respect, even you (quote). Ahmad, if you had saved all those 5 minutes waiting for me, you would have finished your PhD by now. Thank you all.

I would like to thank my friends at Bon Yakhteh, in particular Ehasn, Yousof, Hamid and Shervin for generously helping me with my never ending paper works in Iran, not to mention the great Dizis that we had with Dr. Soleimani and Dr. Karimi in Darakeh.

I would like to thank my parents and my brothers who were there whenever I needed them. I am also really grateful to my parents-in-law and my sister-in-law who have always been very supportive of both Elham and me.

Words cannot thank you Elham. But you know it yourself that without you it would have been impossible. And the journey continues with you…

Ali

Summary

Human amyloid precursor protein (APP) belongs to a gene family that codes for type I trans-membrane proteins in different species, including fruit flies, roundworms and mice. Amyloid Precursor-like Protein1 and 2 (APLP1 and APLP2) are the two other mammalian members of this gene family. Like APP, APLP1 and APLP2 are transmembrane proteins with a large extracellular N-terminal domain and short cytoplasmic C-terminal fragment. Another common feature of APP/APLPs is their processing by enzymatic activities called secretases. Unlike APLP1 and APLP2, APP processing can generate amyloid beta peptides (Abeta) which precipitate in the brain of Alzheimer patients. Therefore, it is not surprising that APP has been extensively studied in the context of Alzheimer disease.

Nevertheless, APP and its two paralogues, APLP1 and APLP2, are expressed in early stages of brain development suggesting a physiological function for these proteins in early neurodevelopment. However, genetic knock-out and shRNA studies have led to contradictory conclusions about their role during embryonic brain development. In particular, down-regulation of APP in precursors and neurons of the developing cortex *in vivo* blocks the migration of neurons towards the cortical plate, while conversely, neurons in an APP/APLP1/APLP2 triple knock-out (ko) mouse over-migrate and accumulate ectopically in the marginal zone. The yet unexplained discrepancy between the over-migration effects in the triple ko and the blocked migration in the case of APP down-regulation shows that the role of the APP family members in the course of cortical development is still unclear.

We hypothesized that APP and APLPs might regulate distinct processes in the developing cortex based on the differential mRNA expression profiles for APP gene family members. During cortical development: APP is found in the cortical plate (CP) and ventricular zone (VZ), APLP2 in the VZ and subventricular zone (SVZ) and APLP1 in the CP only. The restriction of APLP2 expression to the proliferative zones (VZ/SVZ) of the developing cortex suggests a specific function for APLP2 in the development and specification of cortical progenitors. Therefore we focused our attention on the involvement of APLP2 in cortical development.

To this end, we interfered with APLP2 expression in developing cortices of wildtype (wt) mice using *in utero* electroporation. APLP2 down-regulation in wt cortices did not change cortical positioning of neurons. Next, we down-regulated APLP2 expression in APP/APLP1 dko mice reasoning that partial overlapping functions with APP and APLP1 might compensate for the absence of APLP2. While APP/APLP1 dko neurons migrated

normally to the cortical plate, similar to their wt counterparts, further APLP2 down-regulation in APP/APLP1 dko cells blocked cells predominantly in the proliferative zones of the developing cortex, leading to altered cortical positioning. We will use the term "triple ko" in the rest of the thesis to refer to these APP/APLP1dko cells that express APLP2shRNA. Our analysis shows that arrested cells remain undifferentiated as demonstrated by the continuous expression of progenitor and mitotic markers. We find that the morphology of "APP/APLP1/APLP2 triple ko" migrating neurons and "triple ko" radial glia fibers, a major substrate for neuronal migration, is normal. Furthermore, the migration of triple ko neurons *in vitro* seems not affected. Further investigation of the properties of neuronal progenitors showed delayed neuronal differentiation and decreased cell cycle exit of the triple deficient cells.

In summary, our data reveal a novel function of APLP2 in the regulation of proper cell cycle exit of neuronal progenitors.

Samenvatting

Humaan amyloid voorlopereiwit (Amyloid Precursor Protein of APP) behoort tot een genfamilie die codeert voor type I transmembranaire proteïnen in verschillende diersoorten, waaronder fruitvliegen, rondwormen en muizen. Amyloid Precursor-like Proteïne 1 en 2 (APLP1 en APLP2) zijn de 2 andere leden van deze genfamilie in zoogdieren. Zoals APP zijn APLP1 en APLP2 transmembranaire proteïnen met een groot extracellulair N-terminaal domein en een kort cytoplasmatisch C-terminaal fragment. Een andere gemeenschappelijke eigenschap van APP/APLPs is hun klieving door enzymen, de secretasen. Anders dan APLP1 en APLP2, kan het verwerken van APP amyloïd beta peptiden (Abeta) genereren die neerslaan in de hersenen van Alzheimer patiënten. Daarom is het niet verrassend dat APP uitgebreid bestudeerd is in de context van de ziekte van Alzheimer.

APP en zijn 2 paralogen, APLP1 en APLP2, komen al tot expressie in vroege stadia van hersenontwikkeling, wat een fysiologische functie voor deze proteïnen suggereert in prille neuronale ontwikkeling. Genetische knock-out en shRNA studies hebben echter geleid tot contradictorische conclusies betreffende hun rol tijdens embryonale hersenontwikkeling. Meer bepaald down-regulatie van APP in voorlopers en neuronen van de ontwikkelende cortex *in vivo* blokkeert de migratie van neuronen naar de corticale plaat, terwijl omgekeerd, neuronen in een App/Aplp1/Aplp2 drievoudige knock-out (ko) muis over-migreren en ectopisch accumuleren in de marginale zone. De tot nog toe onverklaarde discrepantie tussen de over-migratie effecten in de drievoudige ko en de geblokkeerde migratie in het geval van App down-regulatie toont dat de rol van APP familieleden in het verloop van corticale ontwikkeling nog onduidelijk is.

We veronderstelden dat App en Aplps verschillende processen in de zich ontwikkelende cortex zouden kunnen regelen, gebaseerd op de verschillende mRNA expressie profielen voor leden van de APP genfamilie. Tijdens corticale ontwikkeling komt App tot expressie in de corticale plaat (CP) en de ventriculaire zone (VZ), Aplp2 in de VZ en subventriculaire zone (SVZ) en Aplp1 enkel in de CP. De beperking van Aplp2 expressie tot de proliferatieve zones (VZ/SVZ) van de ontwikkelende cortex suggereert een specifieke functie voor APLP2 in de ontwikkeling en specificatie van corticale voorlopers. Daarom hebben we onze aandacht gericht op de betrokkenheid van Aplp2 in corticale ontwikkeling.

Hiertoe interfereerden we met Aplp2 expressie in de ontwikkelende cortex van wildtype (wt) muizen door middel van *in utero* elektroporatie. Onderdrukken van Aplp2 expressie in

wt cortex veranderde de corticale positionering van neuronen niet. Vervolgens onderdrukten we Aplp2 expressie in App/Aplp1 dubbele ko muizen, met de achterliggende gedachte dat gedeeltelijk overlappende functies van App en Aplp1 zouden kunnen compenseren voor de afwezigheid van Aplp2. Terwijl App/Aplp1 dko neuronen normaal migreren naar de corticale plaat, vergelijkbaar met hun wt tegenhangers, leidt verdere onderdrukking van Aplp2 in App/Aplp1 dko cellen tot stopzetting van hun migratie hoofdzakelijk in de proliferatieve zones van de ontwikkelende cortex. Dit veroorzaakt verandering van hun corticale positionering. Onze analyse toont aan dat afgestopte cellen ongedifferentieerd blijven zoals aangetoond door de continue expressie van voorloper en mitotische markers. We observeerden dat de morfologie van "App/Aplp1/Aplp2" drievoudige ko" migrerende neuronen en "drievoudige ko" radiale glia-vezels, een belangrijk substraat voor neuronale migratie, normaal is. Bovendien lijkt de migratie van drievoudige ko neuronen *in vitro* niet aangetast. Verder onderzoek van de eigenschappen van neuronale voorlopers toont vertraagde neuronale differentiatie en verlaten de cel cyclus van de drievoudig deficiënte cellen minder.

Samengevat brengen onze data een nieuwe functie van Aplp2 in de regulatie van correcte cel cyclus uitgang van neuronale voorlopers aan het licht.

List of Figures

Abbreviations

APP	amyloid precursor protein
APLP	amyloid precursor like protein
APBA1	amyloid precursor protein binding, family A
Arc	Activity regulated cytoskeleton-associated protein
ApoeR2	apolipoprotein E receptor 2
CR	Cajal-Retzius
CASK	calcium/calmodulin-dependent serine protein kinase
CP	cortical plate
CuBD	Copper Binding Domain
Cux1	Cut like homeobox 1
Dab1	Disabled-1
dko	double knock-out
GFAP	glial fibrillary acidic protein
GSK-3	Glycogen synthase kinase 3
FOS	FBJ murine osteosarcoma viral oncogene homolog
GFLD	Growth factor like domain
Ko	Knock-out
LTP	long term potentiation
MAP2	Microtubule-associated protein 2
PCP	planar polarity pathway
PS	Presenilin
KCNH6	Potassium voltage-gated channel, subfamily H (eag-related) member 6
SVZ	Subventricular zone
Tbr2 (Eomes)	T box brain protein 2 (eomesodermin homolog)
VZ	ventricular zone
Vldlr	very low density lipoprotein receptor
Wnt5a	Wingless-type MMTV integration site family, member 5A.

Chapter I: Introduction

Large parts of this chapter have been published in:

S. Ali M. Shariati and Bart De Strooper, Redundancy and divergence in the amyloid precursor protein family, FEBS letters, Volume 587, Issue 13 , Pages 2036-2045, 2013.

In 1987, Amyloid Precursor Protein (*APP*) gene was identified when different research groups were searching for a putative precursor of the Abeta peptide. Amyloid beta peptide was known to be a major component of the amyloid plaques that are found in brains of people with Alzheimer's disease (AD) (Goldgaber, Lerman et al. 1987; Kang, Lemaire et al. 1987; Tanzi, Gusella et al. 1987). In the following years, homologues of APP were identified in human, mouse, as well as in *Drosophila* (Rosen, Martin-Morris et al. 1989; Coulson, Paliga et al. 2000). Although human APP was originally identified and named because of its link to AD, the conservation of the APP genes in the genomes suggests an advantage for the organisms. Therefore, parallel to the effort in understanding the pathogenic role of the human APP in AD, several groups are investigating the physiological function of APP proteins with a particular focus on the nervous system. The work presented in this thesis also aim to address the role of APP proteins in the mouse nervous system.

APP is the most-studied member of the APP protein family. I start the introduction by explaining the features of subdomains of APP as a well-studied member of the family. However, the *APP* gene family in mammals has two other members named *APLP1* and *APLP2* (amyloid precursor-like protein). The interaction between the three genes and potential overlapping functions are complicating factors in analyzing the function of APP proteins in loss of function studies. Therefore, explaining evolution of *APP* gene family would help to understand the common and distinct features of APP proteins among different species.

One of the common features of the APP proteins from different species is their proteolytic processing by membrane proteases called secretases. Secretases cleave APP proteins into different fragments with functions potentially distinct from the full-length APP molecule suggesting different mechanism of action for APP proteins. On the other hand, the expression pattern and interaction network of APP and APLPs seems to be distinct for each member. Insight from knock-out models of *APP* genes in different species shows both distinct and overlapping phenotypes for each member. In the case of murine cortical development, deficiency of App proteins (In the text, mouse proteins are indicated with first capital letter followed by lower cases and human proteins are all capital letters. Genes

follow the same rule but are italicized.) leads to apparently opposite outcomes in neuronal migration. We hypothesized that different members of App proteins can regulate distinct processes during cortical development. In particular, we focused on the role of Aplp2 in the regulation of neural stem cell differentiation.

1.1 Amyloid Precursor Protein domain structure and function

APP is a type I transmembrane protein that has a large N-terminal domain and a short C-terminal fragment. The N-terminal domain of APP is divided into two regions: so called E1- and E2- regions together with two unstructured parts called the acidic and linker regions (Fig. 1). The N-terminal-part of APP starts with the E1 domain which encompasses Growth-factor like domain (GFLD) and a Copper binding domain (CuBD). The crystal structure of GFLD showed that this domain is composed of 9 β stands and 1 α helix which together form a globular compact domain (Rossjohn, Cappai et al. 1999). There are three disulfide bonds in this domains; Cys38-Cys62, Cys37-Cys117, Cys98-Cys105. The Cys98-Cys105 disulfide bond is at the center of a highly charged surface between amino acid 96-110 patch (Rossjohn, Cappai et al. 1999). This sequence was previously shown to form a positively charged surface for heparin binding (negatively charged polysaccharide glycosaminoglycan) that regulates neurite outgrowth in primary neurons (Small, Nurcombe et al. 1994). In fact, combination of APP and heparan sulfate proteoglycan significantly potentiated the neurite outgrowth in primary neurons *in vitro*, whereas separately they did not (Small, Nurcombe et al. 1994). This is particularly interesting because it has been shown that the E1 domain of APP forms dimers upon binding to heparin (Dahms, Hoefgen et al. 2010) suggesting that the dimerization of APP can lead to the generation of a binding site for signaling factors that modulate neurite outgrowth. Alternatively, one can hypothesize that secreted GFLD binds to heparan sulfate proteoglycan in ligand-receptor mode of action, a mechanism that separates the function of soluble APP and membrane anchored APP. However, the functional importance of this interaction and the definitive putative receptor for soluble APP remain unidentified. The GFLD is followed by the CuBD which has 3 β strands and 1 α helix which are connected by two disulfide bonds (Barnham, McKinstry et al. 2003; Kong, Adams et al. 2007). His147, His151 and Tyr168 are the residues that participate in copper binding by the CuBD (Barnham, McKinstry et al. 2003; Kong, Adams et al. 2007). These three residues are conserved in human APLP2 and mouse Aplp2 suggesting a possible conservation of this copper binding between APP and APLP2.

This might provide neurons with a defense system against copper toxicity caused by high concentration of copper. Supporting this hypothesis, *App* knock-out (ko) neurons are more sensitive to neuronal death caused by copper toxicity. Unlike *App* ko neurons, *Aplp2* ko neurons behave similar to the wt in response to high concentration of copper suggesting that the *App* protein regulates indeed copper homeostasis (White, Multhaup et al. 1999). The GFLD and CuBD are linked by an interdomain region that constitutes an interface for the interaction of the two subdomains. Many of the residues that participate in this interaction are conserved between APP and APLP2 but not APLP1 (Dahms, Hoefgen et al. 2010). In addition, Kaden et al showed that a synthetic peptide corresponding to the wt sequence of this loop region reduced dimerization of APP *in vitro*. This reduction was accompanied by a selective decreased β-cleavage of APP suggesting a role for dimerization in the regulation of APP processing (Kaden, Munter et al. 2008). In a cellular context, APP and APLPs promote cellular adhesion by dimerization in COS7 (Fibroblast-like Kidney Cells from monkey) (Soba, Eggert et al. 2005). Soba et al showed that APP and APLPs can form homo- and heteromeric complexes which are dependent on the E1 domain. These interactions at the cell surface increased cell clustering, supporting the intercellular adhesion role for APP and APLPs. Using a cell aggregation assay, *App* ko mouse embryonic fibroblasts formed similar number of aggregates when compared to wt. However, double ko of *App/Aplp1* or single *Aplp2* ko fibroblasts reduced the number of aggregates raising the possibility that App/Aplp1 and Aplp2 do not equally contribute to the fibroblasts adhesion and Aplp2 plays a more significant role (Soba, Eggert et al. 2005).

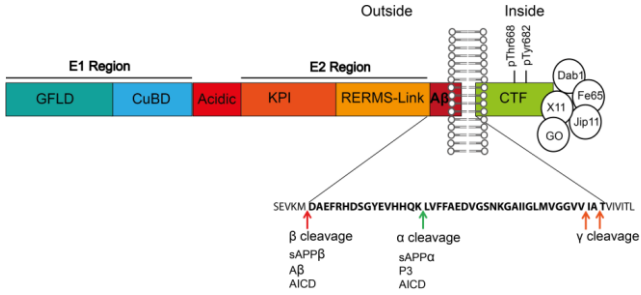

Figure 1. Schematic illustration of APP subdomains

Schematic representation of subdomains of APP. large extracellular domain of APP can be divided into E1 and E2 regions which are two structural subdomains of the APP n-terminal linked by a stretch of acidic amino acid. The E1 region contains growth-factor like domain (GFLD) and a copper binding domain (CuBD). The E1 and the E2 regions are linked by a short stretch of acidic amino acids. Two important features of E2 region are Kunitz protease inhibitor (KPI) and RERMS sequence which may contribute to APP signaling. APP is cleaved next to Abeta region (in red) by β secretase activity (red arrow) leading to production of soluble APPβ. Next APP stub is further processed by γ secretase activity (orange arrows) leading to production of Aβ and APP intracellular domain (AICD). Alternatively, the cleavage of APP can start with α secretase activity (green arrow) which will generate soluble APPα, and P3 and AICD by γ secretase activity. The short C-Terminal of APP is where APP binds to its intracellular interactors which are further discussed in the text. Two well-studied phosphorylation sites are also indicated and further discussed in the text.

An unstructured acidic region links the E1 with the E2 domain. Earlier studies showed that a distinct sequence of five amino acid residues known as the RERMS sequence resides in E2 region which possibly contributes to the growth promoting properties of APP such as fibroblast proliferation, neuronal survival and neurite outgrowth (Fig.1) (Ninomiya, Roch et al. 1993; Jin, Ninomiya et al. 1994; Ninomiya, Roch et al. 1994; Yamamoto, Miyoshi et al. 1994). The crystal structure of E2 domain revealed 6 α helices forming a coiled-coiled structure. The overall structure of E2 domain of APLP1 is similar to APP meaning it can form dimers and bind to heparin (Lee, Xue et al. 2011; Xue, Lee et al. 2011). Analysis of the crystal structure showed that E2 domain dimers can form in anti-parallel orientation. Dimerization of the E2 domain creates a groove of positively charged amino acids that is a binding site for heparin. Unlike the heparin binding site in the E1 domains, the heparin binding residues in the E2 domain are conserved (Wang and Ha 2004). Interestingly, heparin binding induced dimerization of E2 domains (Wang and Ha 2004). Therefore, it is

very likely that these two heparin binding domains regulate the dimerization and binding properties of full-length and soluble APP and APLPs. A third region that can mediate APP dimerization is located at the boundary of transmembrane and juxtamembrane domain of APP where three GxxxG motifs are located. These motifs are situated close to the cleavage sites of APP and therefore might regulate APP processing and function (Kienlen-Campard, Tasiaux et al. 2008; Sato, Tang et al. 2009; Khalifa, Van Hees et al. 2010). Indeed, this GxxxG motif was shown to be required for Abeta production but not APP Intracellular Domain (AICD) release which is the product of γ-cleavage of the APP C-terminal fragment. Changing the glycine pair to leucine significantly decreased the production of Abeta in CHO cells (Kienlen-Campard, Tasiaux et al. 2008). Overall, these data suggest that APP has adhesion properties and may be involved in cell-cell or cell-substrate adhesion and (or) signaling at the surface of the cell (Reinhard, Hebert et al. 2005).

In analogy with Notch signaling, the processing of the APP C-terminal fragment can release AICD which is postulated to form a multi-meric complex with transcriptional activity of cytoplasmic fragment of the APP (Cao and Südhof 2001; Cupers, Orlans et al. 2001). Initially, when intracellular domain of APP was fused to Gal4 transcription factor, this fusion protein alone did not activate the Gal4-dependent reporter. Searching for possible interactors that can boost AICD mediated transcription, over-expression of Fe65 turned out to be a potent stimulator of transcription. Finally, the authors proposed that AICD-Fe65 bind to Tip60, a histone acetyltransferase, to form a nuclear ternary complex that directly regulates transcription (Cao and Südhof 2001). Later, it was shown that cytoplasmic fragment of the APP has a short half life (less than 5 min) and is partially distributed to the nucleus (Cupers, Orlans et al. 2001). More recently, a similar Gal4 assay showed that transcriptional activity of AICD depends on Med12 which is a RNA polymerase II regulator, suggesting a direct link between AICD of APP and the transcriptional machinery. In addition, a physical interaction between AICD and Med12 supported the role of AICD in gene regulation (Xu, Zhou et al. 2011). Until now, many endogenous AICD target genes such as APP itself, BACE1, GSK-3β, KAI1 and Neprilysin were reported using exogenous expression of the AICD (Nalivaeva and Turner 2013). In contrast, complete deficiency of APP/APLPs did not influence the expression of the proposed target genes such as APP, KAI1, GSK-3β and Neprilysin, suggesting that intracellular domains of APP/APLPs are not potently involved in gene regulation at basal level (Hebert, Serneels et al. 2006). Therefore, the reports are conflicting on the transcriptional role and the identity of AICD target genes (Hebert, Serneels et al. 2006; Waldron, Isbert et al. 2008; Huysseune, Kienlen-Campard et al. 2009; Xu, Zhou et al. 2011).

Currently, the experimental data are not available to compare the structure of the APLP2 with APP and APLP1. As it was discussed, the crystal structure of the E2 domain of APLP1 is very similar to APP but the crystal structure of APLP2 or its subdomains are not available. At the level of protein sequence each one of these protein has unique motifs which potentially can confer specific structural features to each member. For example, the N-terminal of APLP2 homologues contains a unique GTGFAVAE motif with unknown function (Fig. 2). On the contrary, the C-terminal YENPTY motif is universally conserved in all members of the APP protein family, providing a common site for intracellular interactors of APP and APLPs (Fig.1,2). Experimental structural data for APLP2 will be valuable in understanding similarities and differences between APLP2 and APP or APLP1.

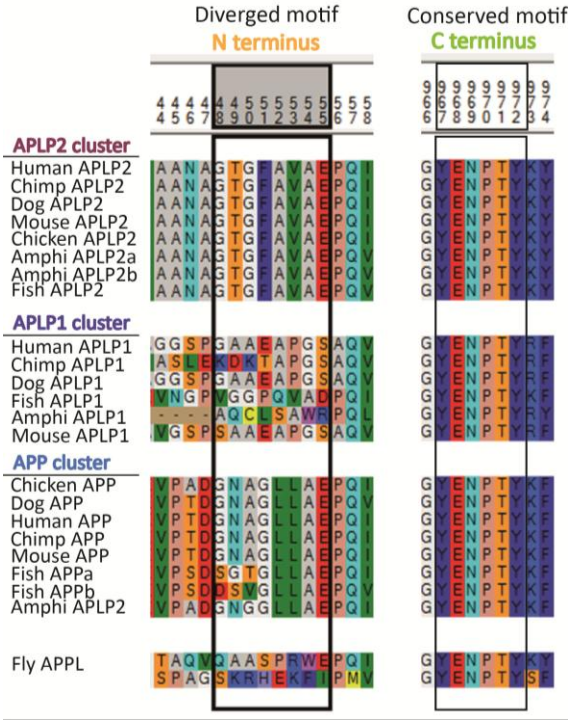

Figure 2. Examples of conserved and diverged motifs in the APP protein family

Alignment of APP protein family shows that the YENPTY motif at the c-terminal of this gene family is a universally conserved motif. The numbers above the figures indicate the alignment position of amino acids from N terminal to C terminal.

1.2 Evolution of the Amyloid Precursor Protein Family

Genes encoding for the APP protein family have experienced several twists and turns during evolution. APP-like proteins have not been identified in prokaryotes, yeasts and plants (Fig. 3). The simplest and earliest branches of the evolutionary tree in which *APP*-like genes have been identified contain insects such as the fruit fly (*D.melanogaster*) and roundworms (*C.elegans*) each carrying one gene encoding for an APP-like protein. It is intriguing that APP-like proteins first emerge in *Bilaterians* with an early nervous system with functional synapses (Emes, Pocklington et al. 2008; Ryan and Grant 2009). Indeed, the extracellular domains of APP molecules have cell adhesion properties and can promote cell-cell adhesion (Soba, Eggert et al. 2005). Such intercellular interaction is important in early evolution for the generation of the synaptic junction (Emes, Pocklington et al. 2008; Emes and Grant 2012). Strikingly, when over-expressed in HEK cells, APP can potently induce synaptogenesis in the contacting axon and this activity requires the extracellular domain as well as the intracellular part of APP. The latter associates with presynaptic molecules such as APP binding family A (APBA1) and Calcium/calmodulin-dependent serine protein kinase (CASK) (Wang, Wang et al. 2009). Interestingly, APP is required both at pre- and postsynaptic compartments to induce synaptogenesis (Wang, Wang et al. 2009) which suggests that ancestral APP indeed might have evolved as a transmembrane protein responsible for homophilic interactions at the synaptic junction.

Five nodes of duplications are observed in the phylogenetic tree of the APP protein family when using Ensemble comparative genomics tools (simplified in Figure 3). For example, zebra fish (*D.rerio*) has in total four genes encoding APP proteins: two homologues for the human *APP* gene (*appa* and *appb*) plus *aplp1* and *aplp2* (Fig. 3). Similar to fishes, amphibians (*Xenopus laevis*) carry four *app* genes in their genome but they have two homologues for the human *APLP2* gene: *aplp2a*, *aplp2b* plus *app* and *aplp1* (Fig. 3). Instead, birds (*G. Gallus*) have lost the *APLP1* gene leaving them with *APP* and *APLP2* genes (Fig. 3). The paradoxical expansion and contraction of the APP family suggest that the duplications of the encoding genes have been the subject of highly selective evolutionary forces. The complicated trajectory of the evolution of the APP protein family ends with the three well-studied members in mammals: *APP*, *APLP1* and *APLP2* (Fig. 3) (Zheng and Koo 2011).

The evolutionary maintenance of a duplicated gene in the genome is influenced by the accumulation of genetic mutations affecting the function of the descendant duplicates. Three possible outcomes of duplication have been proposed: non-functionalization, neo(sub)functionalization or increased gene dosage (Innan and Kondrashov 2010). The non-functionalization scenario is the result of the accumulation of deleterious mutations leading to pseudogenization (Ohno 1970). In case of neo-functionalization, mutations confer new features to the duplicate which leads to the acquisition of new functions distinct from the ancestral ones (Force, Lynch et al. 1999). Subfunctionalization is a modified version of neofunctionalization in which the function of the ancestral gene becomes subdivided into subfunctions for each duplicate providing cells with proteins with more specialized functions (Innan and Kondrashov 2010). Finally without any functional innovation, duplication can provide cells with genetic robustness and redundancy by increasing the gene dosage for dosage sensitive genes (Conrad and Antonarakis 2007).

Which model of gene duplication and evolution can be applied to the APP family? While the prevalent vision stresses the "increased gene dosage", as I will discuss, more in depth interpretation of the data provide supporting evidence for neo or subfunctionalization of APP and APLPs as well.

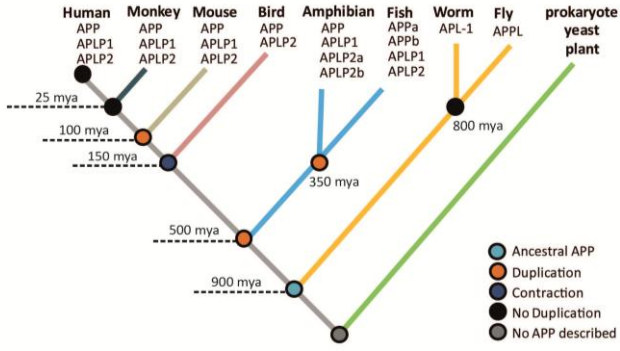

Figure 3 Simplified dendrogram of APP protein family

The tree illustrates the important events in the evolution of APP gene family. The duplication and contraction nodes are color coded. The lengths of the lines are not proportional to the evolutionary distance of species. For details of APP protein family evolution see the text. Mya: million years ago.

1.3 Proteolytic processing of Amyloid Precursor Proteins

APP undergoes proteolytic processing by enzymatic activities of the membranes secretases known as: α- secretase, β-secretase, and γ-secretase (Fig. 1). The first cleavage of APP by α-secretase or β-secretase releases soluble fragments and generates a membrane bound C-terminal stub which is the substrate of γ-secretase. In case of APP this proteolytic processing is classified into two pathways: amyloidogenic when β-secretase cleaves first and non-amyloidogenic when α-secretase cleaves first. Only the former is linked to the pathogenesis of Alzheimer's disease because it produces Aβ which is the main component of amyloid plaques in the brain of patients. Interestingly, the proteases α-secretases, β-secretases and γ-secretases are largely conserved during evolution, together with the overall processing of the membrane bound APP paralogues resulting in the release of APP ectodomain and intracellular domain.

α-secretase cleaves APP between Lys16 and Leu17 of the Abeta domain (Fig. 1) (Esch, Keim et al. 1990). Cleavage of APP by α-secretase activity results in generation of a soluble fragment (sAPPα) and an 83 amino acid C-terminal fragment (C83). In mammals, different members of the "A disintegrin and metalloprotease" (ADAM) family have been proposed as α-secretases (Deuss, Reiss et al. 2008; Vingtdeux and Marambaud 2012). Over-expression of ADAM 9, 10, 17 in cell culture system can change the processing of APP (Deuss, Reiss et al. 2008; Vingtdeux and Marambaud 2012). In neurons, there is convincing evidence that Adam10 carries out the constitutive processing of APP (Jorissen, Prox et al. 2010; Kuhn, Wang et al. 2010). The secretion of sAPPα is reduced by 90% in Adam10 deficient neurons, reflecting the importance of Adam10 for constitutive processing of App. ADAM10 and ADAM17 can both cleave APLP2, but, similar to APP, ADAM10 was found to be a constitutive α-secretase of APLP2 in neurons (Hogl, Kuhn et al. 2011). Although ADAM10 constitutively processes APP and APLP2, the induced processing of APP and APLP2 are regulated differently. Insulin growth factor 1(IGF1) has been shown to induce production of sAPPα, sAPLP1 and sAPLP2 (Adlerz, Holback et al. 2007). Different α-secretases mediate the effect of IGF1 induced secretion of soluble fragments of APP and APLPs (Jacobsen, Adlerz et al. 2010). ADAM10 inhibition blocks the effect of IGF1 induced shedding of APP. Likewise, APLP1 processing was blocked after inhibition of ADAM10, whereas APLP2 ectodomain shedding was decreased only after down-regulation of ADAM17 (Jacobsen, Adlerz et al. 2010). Thus, it is likely that ADAM17 mediates the induced processing of APLP2, whereas ADAM10 mediates its constitutive processing.

In flies, a 130KD fragment that misses the carboxyl terminus of full-length APPL was identified and proposed as secreted form of APPL (Luo, Martin-Morris et al. 1990). Kuzbanian is the α-secretase like protease of flies which was identified because of its role in regulation of Notch signaling and neurogenesis (Rooke, Pan et al. 1996). More recently it was shown that Kuzbanian can indeed also affect processing of flies' APPL (Carmine-Simmen, Proctor et al. 2009). Therefore, despite small differences, α-secretase cleavages are conserved in APLPs family reflecting the physiological importance of this pathway. The physiological importance of this pathway is supported by data showing that sAppα can rescue the phenotypes of App deficient mice (Ring, Weyer et al. 2007). One can speculate that α-secretase generates soluble ectodomains of APP and APLPs which are active ligands for unknown receptors. It is not clear to what extent the biological activity of sAPPα, sAPLP1α and sAPLP2α are different or similar. One example of similar activity of different soluble fragments of APP family is neurite outgrowth. It has been shown that the conditioned medium of CHO cells over-expressing APP, APLP1 or APLP2 similarly increase the length of the axons, suggesting similarity in the function of the soluble fragments of APP and APLPs in neurite outgrowth regulation (Young-Pearse, Chen et al. 2008).

β-secretase activity cleaves APP next to the N-terminal of the Abeta region generating sAPPβ and C99 (Fig. 1). Bace1 was identified as the major neuronal enzyme with β-secretase activity and its deficiency blocks Abeta secretion in neurons (Vassar, Bennett et al. 1999; Cai, Wang et al. 2001). The decreased level of Abeta is accompanied by reduced levels of β-CTF (Pastorino, Ikin et al. 2004). It has been shown that BACE1 can alter processing of APLPs when over-expressed in HEK293 cells (Li and Südhof 2004). Consistent with these data, Bace1 deficiency significantly decreased levels of soluble Aplp2 in the brain, whereas its over-expression had opposite effect (Pastorino, Ikin et al. 2004). Parallel to decreased soluble Aplp2 in brain, the β-CTF of Aplp2 was diminished in Bace1 ko brain reflecting the overall similarity between App and Aplp2 β-cleavage (Pastorino, Ikin et al. 2004). However, more recently it was shown that Bace1 ko did not alter the CTF production of App or Aplps raising the possibility of compensatory mechanisms for β-secretase activity (Sala Frigerio, Fadeeva et al. 2010). β-secretase activity has also been described in flies and interestingly dBACE can process APPL into neurotoxic Aβ like peptide which can deposit in the brain similar to human APP (Carmine-Simmen, Proctor et al. 2009). Therefore, similar to α-cleavage, β-secretase activity is conserved in the APP protein family. As it will be discussed later (section 1.3.3), the 16 amino acid difference between sAPPα and sAPPβ have been proposed to contribute to striking differences in their functions, although this needs further mechanistic insight.

In addition to ectodomain shedding, β-secretase proteolytic processing of APP results in the release of Aβ with high propensity to aggregate. As this is a continuous process, the brain is constantly faced with the challenging task of controlling the concentration of Aβ below the aggregation threshold. The Aβ region is a novel feature of the APP paralogue and is not present as such in APLP1&2 or in the APP-homologues in *C. elegans* (APL1) and *D. melanogaster* (APPL). For example, recently it was shown that sequential cleavage of APLP1 by β- and γ-secretases generate APLP1 β peptides of 25, 27 and 28 amino acids (to be compared with Aβ from APP that contains 38,40 and 42 amino acids) which do not aggregate in the brains (Yanagida, Okochi et al. 2009). Interestingly, the analogy between the processing of APP and APLP1 has raised the possibility of using APLP1 derived p28 in the cerebrospinal fluid as a surrogate marker to detect altered activity of γ-secretases in individuals with an increased risk of Alzheimer 's disease (Yanagida, Okochi et al. 2009).

γ-secretase cleaves C99 and C83 generated by β-cleavage and α-cleavage respectively (Fig. 1). γ-secretase is a multi-subunit complex that is composed of : Presenilins (PS1 and PS2), anterior pharynx-defective 1 (APH-1), Nicastrin, and Presenilin enhancer protein 2 (Pen2). A series of genetic and biochemical experiments showed that PS1 deficiency leads to the accumulation of APP CTF and blocks Abeta production in neurons showing that PS1 is the catalytic subunit of γ-secretase (De Strooper, Saftig et al. 1998; De Strooper, Annaert et al. 1999; Wolfe, Xia et al. 1999). Likewise, the CTFs of APLP1 and APLP2 are also cleaved by γ-secretase. One of the early hints showing that APLPs are also processed by γ-secretase came from accumulation of APLP1 CTF in PS1 deficient neurons (Naruse, Thinakaran et al. 1998). Further, CTFs of APLP1 and APLP2 were accidentally co-purified together with APP CTFs suggesting that APLP1 and APLP2 are also cleaved by γ-secretase (Gu, Misonou et al. 2001). Using both chemical inhibition and genetic deletion, there is now compelling evidence for γ-secretase processing of APLP1 and APLP2 (Walsh, Fadeeva et al. 2003; Eggert, Paliga et al. 2004; Yanagida, Okochi et al. 2009).

Processing of C99 or C83 by γ-secretase generates AICD, in other words AICD is the common product of both amyloidogenic and non-amyloidogenic pathways. As it was discussed, this fragment can potentially translocate to nuclei to regulate gene expression. This model postulates that APP works as a receptor that mediates signals from membrane to nucleus in a process known as Regulated Intramembrane Proteolysis (RIP) (Brown, Ye et al. 2000). For example, the extracellular domain of APP has been shown to bind to Tag1 in a ligand-receptor model and Tag1-APP binding can regulate neurogenesis. Enhanced neurogenesis of Tag1 deficient neuronal stem cells was reversed after expression of AICD. Instead, AICD with a mutation in the Fe65 binding site could not rescue the neurogenesis

defect, supporting a signaling role for the AICD-Fe65 complex (Ma, Futagawa et al. 2008). Similar fragments of APLP1 and APLP2 (named ALID1 and ALID2) can also potentially contribute to gene expression regulation (Scheinfeld, Ghersi et al. 2002); however evidence for their role in neurogenesis and signaling function is lacking, and, as discussed above, we could not show a direct signaling role of the AICD (Hebert, Serneels et al. 2006).

1.4 Temporally and tissue specific regulated expression of APP family proteins

One of the determinants of the function of gene duplicates is the modification of their regulatory proximal elements which can lead to transcriptional divergence. In turn, transcriptional divergence is likely to result in diversification of the duplicates with refined functions instead of redundant functions. In particular, genetic differences in regulatory elements of genes that are expressed in the developing cortex have been proposed as a primary force influencing the emergence of mammalian brains (Johnson, Kawasawa et al. 2009; Zhang, Landback et al. 2011). Members of the APP family proteins are characterized by a specific tissue expression during different developmental stages which is important to keep in mind when discussing physiological functions.

The genomes of *D.melanogaster* and *C. elegans* each contain only one APP "like" gene. APl-1 of *C.elegans* is expressed in neurons mainly and also in a few other cell-types (Hornsten, Lieberthal et al. 2007). Expression of APPL in *D.melanogaster* is restricted to the nervous system (Rosen, Martin-Morris et al. 1989). In mammals Aplp1 shows a neuron specific expression whereas App and Aplp2 are expressed by various cell types. Until recently, App was assumed to be expressed by all cell types in the brain (Lorent, Overbergh et al. 1995). However, using a specific antibody to stain App , Guo et al showed that App is expressed predominantly in neurons in the adult brain (Guo, Li et al. 2012). At the transcript level, a complementary pattern has emerged from *in situ* hybridization analysis of APP and Aplps transcripts during development of the cortex. Lopez-Sanchez et al have demonstrated that Aplp1 expression is restricted to the post-mitotic cortical plate during cortical development while Aplp2 transcripts show a specific distribution in the proliferating neurons in the ventricular and/subventricular zone. App finally appears both in the ventricular zone neurons as well as in post-mitotic neurons of the cortical plate, thus

showing partial overlapping expression with both Aplp1 and Aplp2 (López-Sánchez, Müller et al. 2005). The same expression pattern is demonstrated in publicly available atlases of the developing cortex, including Genepaint and Eurexpress (Visel, Thaller et al. 2004; Diez-Roux, Banfi et al. 2011). The region specific expression pattern of App, Aplp1 and Aplp2 suggests functional specialization of each member during different stages of neuronal development.

The genetic factors that contribute to the transcriptional divergence of APP duplicates are not well defined. However, analysis of the proximal element of APP and APP like proteins in different species reveal a CAGA box within the APP 5′-UTR which is not present in APP like proteins (Maloney, Ge et al. 2004). This CAGA box might regulate expression of APP in response to signaling pathways such as TGFβ (Maloney, Ge et al. 2004). More studies are needed to understand the contribution of the proximal element that control regulated expression of APP and APLPs.

1.5 The interaction networks of APP, APLP1 and APLP2 show specificity

Proteins are part of the dynamic networks of interactions that can show cell type and tissue specificity (Bossi and Lehner 2009). Evolutionary changes in specificity and strength of these interactions impact the function of the proteins and their networks (Robertson and Lovell 2009). Rewiring of the interaction network of paralogous protein is a clear sign for their functional refinement (Robertson and Lovell 2009).

Several binding partners have been proposed for APP with various functional implications. (Reinhard, Hebert et al. 2005; Zheng and Koo 2011; Guo, Wang et al. 2012). In general, the interactors of APP can be divided into two groups: extracellular binders with a possible ligand mode of action and intracellular binders with a possible signaling mode of action. F-spondin, Tag1, Reelin, Netrin, Lingo-1, Pancortins are among extra-cellular binders while Fe65, JIP, JNK, Mint1/X11, Dab1 are among intracellular binders of APP (Reinhard, Hebert et al. 2005; Zheng and Koo 2011; Rice, Townsend et al. 2012). If APP and APLPs share similar biological functions, then they are expected to be part of a similar protein network. However, a study by Bai et al challenges this idea by showing different networks of interaction for App and Aplps (Bai, Markham et al. 2008). Unexpectedly, a systematic comparison of *in vivo* brain interactome of App, Aplp1, and Aplp2 revealed different sets

of interactors for each paralogue. A significant proportion of identified App interactors were consistent with previously published data supporting the reliability of candidate interactors. Surprisingly, the combined analysis of interactors shows only one interactor in common; Ras GAP-activating like Protein 1 which binds to both Aplp1 and Aplp2 (Bai, Markham et al. 2008). In this study, ER chaperones populated the interaction network of App. Over-representation of ER chaperone in the App interaction network suggest sensitivity of App folding, the distinct interaction of Aplp2 with Rho family of GTPases such as RhoA and RAC1 can link APLP2 to G-protein signaling pathway (Bai, Markham et al. 2008).

It is likely that the difference in the interaction networks of App and Aplps arises from different subcellular localization of each paralogue or from their differential expression pattern (Huminiecki and Wolfe 2004; Kaden, Voigt et al. 2009). Indeed, using fluorescent tagged version of APP and APLPs in HEK293 cells, Kaden et al showed that APLP1-YFP is primarily localized to the cell surface. Most of the APP-YFP was found in intracellular compartments such as the ER and endosomes and to a lesser extent in the Golgi apparatus. APLP2 was equally distributed at the cell surface and intracellular compartments showing partial overlapping localization with both APP and APLP2 (Kaden, Voigt et al. 2009). These findings suggest that in part specificity of interaction network of APP and its paralogues can be due to their differential subcellular localization. Regardless of the reason for different set of interactors, the specificity of interaction networks for APP duplicates can contribute to the separation of their role in different cellular contexts.

1.6 Various roles of the APP members in the nervous system

Loss of function studies is still the standard approach to deduce the physiological role of a gene. The APP family has been covered rather well in that regard with knock-outs (ko) in *D. melanogaster*, *C. elegans* and several combinations of gene ko in *M. musculus*. The data are somewhat divergent, but overall they suggest strongly a role of the *APP* family in the central nervous system.

1.6.1 The extracellular domain in development of C. elegans

In *C. elegans*, the single gene encoding for members of the *APP* family, is called *apl-1*. The encoded protein is very similar to the mammalian counterpart with a large

extracellular and a short intracellular domain, while the Abeta sequence as such is lacking in APL-1 (Daigle and Li 1993). Loss of APL-1 leads to a molting defect resulting in developmental lethality (Hornsten, Lieberthal et al. 2007). In addition, the a*pl-1* null mutant worms are hypersensitive to the acetylcholinesterase inhibitor aldicarb supporting a role for APL-1 protein at synaptic junctions (Wiese, Antebi et al. 2010). Many reports stress the importance of the conserved intracellular domains of the APP family for its function, but, unexpectedly, the conserved carboxyl terminus fragment of APL-1 is not involved in the phenotype as demonstrated by rescuing the lethality of the *apl-1* null mutant by c-terminus truncated version of APL-1 (Hornsten, Lieberthal et al. 2007). In contrast, the extracellular domain of APL-1 is sufficient to rescue both the lethality and hypersensitivity phenotypes. As this domain is soluble, these data suggest a receptor for APL-1 ectodomain, and indicate the importance of this domain in development (Hornsten, Lieberthal et al. 2007).

1.6.2 D. melanogaster's APPL in axonal wiring and synaptic function

Like *C.elegans, D. melanogaster* also carries one homologue of the *APP* gene, called *Appl*. *Appl* expression is first seen in developing neurons during axogenesis (Luo, Martin-Morris et al. 1990). Flies with an *Appl* null mutation are viable and fertile, but show subtle phenotypes. For instance at the neuromuscular junction *Appl* null mutant flies have a reduced number of neuromuscular buttons, whereas larvae over-expressing APPL show an increased number of buttons (Torroja, Packard et al. 1999). Interestingly, Torroja et al showed that APPL is transported to synaptic buttons and a highly conserved cytoplasmic YENPTY motif of APPL is required for promoting synapse formation (Torroja, Packard et al. 1999). This synaptogenic property might be mediated through interaction of Fasciclin II with APPL, while APPL is binding via its conserved cytoplasmic domain to APPBA1 at the synapse (Ashley, Packard et al. 2005). APPL has also been implicated in regulation of neurite arborization (Leyssen, Ayaz et al. 2005). Leyssen et al (Leyssen, Ayaz et al. 2005) showed that both APPL and its human homologue APP can promote post-developmental neurite arborization in D. melanogaster. Similar to its synaptogenic role, APPL requires the conserved cytoplasmic YENPTY for its effects on neurite arborization, but this time the signal is transduced through the Abelson tyrosine kinase (Abl) pathway (Leyssen, Ayaz et al. 2005). These data suggest a role for APP in the structural plasticity of neurons, whereas in pathological condition such as brain injury APPL might promote neurite arborization (Leyssen, Ayaz et al. 2005). Recently, it was shown that loss of APPL induced a developmental defect in the axonal outgrowth in mushroom bodies of D. melanogaster.

Heterozygosity for Abl kinase significantly enhanced the axonal phenotype of Appl mutant flies. Mechanistically, APPL turned out to interact with core components of the planar polarity pathway (PCP) mediating the WNT5a induced phosphorylation of Disheveled. Thus, it was suggested that modulation of PCP pathway by neuronal APPL might regulate developmental axonal wiring in mushroom bodies (Soldano, Okray et al. 2013). Overall, the loss of function experiments in flies and worms suggest that the ancestral *App* like gene has evolved to serve in the nervous system, in particular in synapse formation and function. While the *Drosophila* counterpart is really a nervous system protein, the situation in *C.elegans* is not completely clear. Absence of APL-1 results in multiple developmental defects for instance decreased body size, and egg-laying rate (Ewald, Raps et al. 2012). It is uncertain whether these phenotypes are the result of defects in the neuronal system or indicate that APL-1 also operates in other cells, and that its function is context-dependent.

1.6.3 The APP family in M. musculus

The situation in mammals is even more complex. The different functions proposed for APP and its paralogues are not converging to a concrete model for APP family function. The single *App* KO mice are viable but show various subtle phenotypes such as 15-20% reduced body weight, disturbed forelimb strength and reduced locomotor activity (Zheng, Jiang et al. 1995). The interpretation that *App* ko mice show subtle phenotypes because of compensation by other APP members is not supported by expression studies of the other members of the APP family: compensatory up-regulation of *Aplp1* and *Aplp2* transcripts were not observed in these mice (Zheng, Jiang et al. 1995).

The alterations in muscular strength and decreased locomotor activity in the *App* null mutant mice might reflect the synaptic role of App in the central nervous system. Immunocytochemical analysis of *App* null mice revealed age-dependent increased glial fibrillary acidic protein (Gfap) immunoreactivity indicating gliosis and reduced staining for synaptic marker markers such as synaptophysin, synapsin and MAP-2 (Dawson, Seabrook et al. 1999). In addition, the mice showed impaired LTP recording which was highly correlated with gliosis (Dawson, Seabrook et al. 1999). Consistent with a defect in LTP, *App* null mutants mice spend more time finding the hidden platform in the Morris water maze test (Dawson, Seabrook et al. 1999) further suggesting a role for App in spatial learning. The defect in LTP was associated with attenuation of GABA-mediated inhibitory post-synaptic currents (Seabrook, Smith et al. 1999). Increased expression of calcium channel, Cav1.2, was suggested as potential mechanism regulating GABAergic synaptic

activity in inhibitory neurons (Yang, Wang et al. 2009). Further experiments will clarify the role of APP in synaptic plasticity of excitatory vs. inhibitory neurons.

Mixed results were obtained on the role of App in the formation of dendritic spines (Bittner, Fuhrmann et al. 2009; Lee, Moussa et al. 2010; Jung and Herms 2012). Bittner et al (Bittner, Fuhrmann et al. 2009) used *in vivo* two photon imaging to show that γ-secretase inhibition reduced spine density in an App dependent manner. In their study layer III and Layer V cortical neurons of *App* ko mice showed a two fold increase in the number of dendritic spines (Bittner, Fuhrmann et al. 2009). In contrast, Lee et al (Lee, Moussa et al. 2010) used primary rat hippocampal neurons to show that down-regulation of App decreases the number of spines, whereas over-expression of App has the opposite effect. App needs both its extracellular and intracellular domains to mediate these effects. Golgi staining of spines of CA1 pyramidal neurons and layer II/III cortical neurons revealed a significant decrease in density and length of spines in *App* null mutants, confirming the role of APP in spine formation *in vivo* (Lee, Moussa et al. 2010). More recently, Tyan *et al* (Tyan, Shih et al. 2012) reported decreased spine density in primary neuronal culture of *App* null mutant mice confirming further the role of App in promoting spine formation. The discrepancy may arise from different methodology used to image the spines (Golgi staining vs. *in vivo* two photon imaging) or analyzing different types of neurons, i.e. deep layer vs. upper layer pyramidal neurons of cortex or CA1 neurons of hippocampus. One can speculate that the effect of APP is cell type specific and age dependent, which remains intellectually an unsatisfying explanation, as it brings little insight into the real function of App. It is interesting however that the role of App in the regulation of neurite formation is reminiscent of the role of *D. melanogaster* APPL in regulation of arborization of neurites. Clearly, there would be some conservation of this role in evolution (Leyssen, Ayaz et al. 2005; Soldano, Okray et al. 2013).

It is noteworthy that the some of the phenotypes reported for App single ko mice are not observed in single Aplp1 and Aplp2 ko mice. Although both Aplp1 and Aplp2 single ko mice are much less extensively analyzed than the *App* knock-out, their phenotypes do not overlap (Heber, Herms et al. 2000). *Aplp1* ko mice appear normal both in forelimb strength and in reduced locomotor activity (Heber, Herms et al. 2000). Similar analysis of dendritic spines of *Aplp2* null mutant neurons did not reveal alterations of spines in those mice (Midthune, Tyan et al. 2012). A very different example of non-conserved function is the feroxidase activity in App which is mediated by the REXXE motif in the extracellular domain of App and is not found in Aplp1 and Aplp2 (Duce, Tsatsanis et al. 2010). Thus, although further work is needed, the different phenotypes of the single ko mice support the

idea that App is specialized in its functions at the synaptic junction, which are likely not fully compensated by Aplp1 and Aplp2. Lack of overt phenotypes in *Aplp1* and *Aplp2* single KO mice does not exclude phenotypes that have escaped scrutiny at this moment.

Combinations of the genetic deletions of App/Aplps have been generated (Heber, Herms et al. 2000; Guo, Wang et al. 2012). Expression of *Aplp2* alone is sufficient for survival of the mice meaning that double deletion of *App* and *Aplp1* is viable. However, in the absence of *Aplp2*, mice can survive only if they express both *App* and *Aplp1*. The viability of *Aplp2* single ko might indicate that App and Aplp1 can work together to compensate for a function that is dominated by Aplp2. At first glance, compensation by *App/Aplp1* together for *Aplp2* deficiency is a possibility, however, no compensatory up-regulation of *App* or *Aplp1* was detected after deletion of *Aplp2* (Heber, Herms et al. 2000; Aydin, Filippov et al. 2011). Alternatively, it is equally possible that APP family independent mechanisms are compensating for the lack of *Aplp2*. Indeed, combination of *Aplp1* ko with *App* or *Aplp2* leads to different outcomes which is survival or lethality showing specificity in function of App and Aplp2 (Heber, Herms et al. 2000).

Several studies support a role for App at the neuromuscular junction. A phenotype emerges only when *App* deletion is analyzed in an *Aplp2* ko background (*App/Aplp2* dko). These mice show reduced vesicle density in presynaptic active zone, excessive nerve terminal sprouting and aberrant apposition of presynaptic and postsynaptic markers indicating a key role for proper formation of synaptic structures at the neuromuscular junction (Wang, Yang et al. 2005; Yang, Gong et al. 2005). Interestingly, proper development of the neuromuscular junction requires App and Aplp2 in the presynaptic motor neurons and the post-synaptic muscles suggesting a transynaptic homophilic or heterophilic interaction between App and Aplp2 (Wang, Wang et al. 2009). In contrast to the prominent role for soluble App in C.*elegans*, expression of soluble App β (sAppβ) in the *App/Aplp2* dko mice (*App/Aplp2 sAppβ*$^{ki/ki}$) did not rescue the lethality or neuromuscular defects of *App/Aplp2* dko mice (Li, Wang et al. 2010). Strikingly, expression of soluble App α (sAppα) rescued the lethality of the *App/Aplp2* dko mice (*App/Aplp2 sAppα*$^{ki/ki}$) (Weyer, Klevanski et al. 2011) indicating that a few amino acid between α and β cleavage are instrumental in the biological function of sApp. However, *App/Aplp2 sAppα*$^{ki/ki}$ showed a widened end plate, impaired neuromuscular transmitter release, and structural abnormalities at the neuromuscular synapses correlating with decreased grip strength. In the central nervous system, the mice showed impaired LTP accompanied with impaired spatial memory (Weyer, Klevanski et al. 2011). Thus, sAppα was not able to rescue several neurological

phenotypes, implying that full length APP is needed. Interestingly, *App/Aplp2 sAppα*$^{ki/ki}$ mice did not have any spine or morphological defects in cortical or hippocampal neurons.

It is very likely that the developmental function of App at the neuromuscular junction is mediated through its highly conserved YENPTY motif in its carboxyl terminus domain since expression of App with a single Tyr(682) to Gly (682), Y682G, mutation in an *Aplp2* null background, leads to the lethality and neuromuscular defects similar to *App/Aplp2* dko mice (Barbagallo, Wang et al. 2011). This conserved Tyr (682) residue is both a docking site for several cytoplasmic partners and regulates processing of App. For example, a significant 15 fold increase in sApp-α together with a 3.5 fold decrease in sApp-β was detected in brain tissue from *App*$^{Y682G/Y682G}$ mice, highlighting the importance of this residue in regulating App processing (Barbagallo, Weldon et al. 2010). NGF-TrkA signaling was proposed as the pathway responsible for regulating the phosphorylation of Tyr(682) of App. Tyrosine phosphorylation of APP was induced after NGF treatment of primary hippocampal neurons. The tyrosine kinase activity of TrkA receptor may mediate the NGF induced tyrosine phosphorylation of App (Matrone, Barbagallo et al. 2011). Further, it was shown that Y682G mutant neurons are insensitive to trophic activity of NGF, suggesting that phosphorylation of Tyr(682) can work down-stream of NGF-TrkA signaling to mediate the trophic effect of NGF. A nearby conserved phosphorylated Thr(668), part of pSer/Thr-Pro motif, is a docking site for Pin1 and this interaction can down regulate production of Aβ peptide from APP (Balastik, Lim et al. 2007). Pin1 is a unique peptidyl–prolyl cis/trans isomerase that can catalyse cis/trans isomerization of pSer/Thr-Pro motifs (Lu 2004). Binding of Pin1 to the pThr 668-Pro motif in c-terminus of App was shown to accelerate its isomerization leading to conformational changes in the c-terminus of App (Pastorino, Ikin et al. 2004). In contrast to the instrumental role of Tyr(682) for survival of the mice during development, mutation in Thr(668) of App (T668A), in an *Aplp2* null background, does not cause lethality or neuromuscular defects (Barbagallo, Wang et al. 2011) highlighting further the importance of tyrosine phosphorylation of APP during development.

The fact that several loss of function App phenotypes emerge in an *Aplp2* null background is evidence for genetic interaction but does not directly address the question whether there is really functional redundancy between these different paralogues. Further thorough analyses of the single mutant mice should be more informative to identify pathways in which the specific effects of Aplps become apparent. Indeed the question remains whether double mutants generate more severe phenotypes either because of complete loss of a redundant function or because of disruption of multiple independent pathways.

Transcriptional profiling of the different single mutant mice supports the second possibility. The transcriptomes of *App* and *Aplp2* single ko mice reveal different sets of genes (Aydin, Filippov et al. 2011). From the 1061 genes that are up or down regulated after deletion of *Aplp2*, only 181 are also found altered in App mutant mice (Yang, Turner et al. 1998; Aydin, Filippov et al. 2011). For instance, signalling molecules that regulate early response to synaptic activity such as KCNH6 (Erg2), Fos and Arc are significantly down-regulated in App mutant mice, but not in *Aplp2* mutant mice, strengthening the evidence for APP function in synaptic plasticity. p21 is such an example that is down regulated in both APP and APLP2 ko mice cortices. p21 is a cyclin-dependent kinase inhibitor that regulate cell-cycle progression during G1 phase of cell cycle. Deficiency of p21 decrease cell cycle exit and enhances proliferation of neural stem cells by regulating the expression of pluripotency factor Sox2 (Kippin, Martens et al. 2005; Marqués-Torrejón, Porlan et al. 2013). Thus, down-regulation of p21 in both *App* and *Aplp2* null mutants may enhance the proliferation of neural stem cells. This is consistent with data from Lopez-Sanchez et al (López-Sánchez, Müller et al. 2005) that demonstrate *Aplp2* transcripts is predominantly enriched in the proliferative zone of the developing cortex while *App* show a partial overlapping expression with *Aplp2* in this area. Soluble fragment of APP and Aplp2 can promote proliferation of EGF expressing progenitors of subventricular zone (Caillé, Allinquant et al. 2004). Thus, overall it appears that App and Aplp2 indeed are partially redundant in neurogenic niches. Nevertheless, it should be noted that next to shared pathways between APP and APLP2, significant differences in the transcriptional response of App or Aplp2 deletion suggest that there are distinct pathways that are regulated by either App or Aplp2. It also of note that it is not yet clear whether expression changes in *App* and *Aplp2* ko mice is due to direct transcriptional activity of intracellular domain of this protein or an indirect effect of loss of those proteins (Cao and Südhof 2001; Hebert, Serneels et al. 2006; Aydin, Filippov et al. 2011).

1.7 A role of APP and APLPs in cortical development?

The mammalian cortex has expanded rapidly across different species and this is associated with the evolution of neocortical related behaviour such as perception and cognition (Kriegstein, Noctor et al. 2006). The expansion of cortical surface is believed to underlie the transition from a smooth cortex (lissencephaly) to a highly folded cortex (gyrencephalic). Indeed, changes in the proliferative pattern of ventricular zone resident neural stem cells have been titled as " a giant leap for mankind " referring to the expanded surface of the human cortex due to more proliferative radial units (Rakic 1995). However,

neural progenitors outside the ventricular zone, for example the intermediate progenitors in subventricular zone, might be as well important players in the evolutionary expansion of the cortex (Kriegstein, Noctor et al. 2006; Fietz and Huttner 2011). In mice, APP and APLP2 are expressed in both ventricular and subventricular neurogenic niches of the developing cortex, and the question whether they play there a role in the development and evolution of the cortex is attractive (Lorent, Overbergh et al. 1995; López-Sánchez, Müller et al. 2005). Recently, several studies support such a role of the APP protein family in both migration and differentiation of neuronal precursor and progenitors during cortical development.

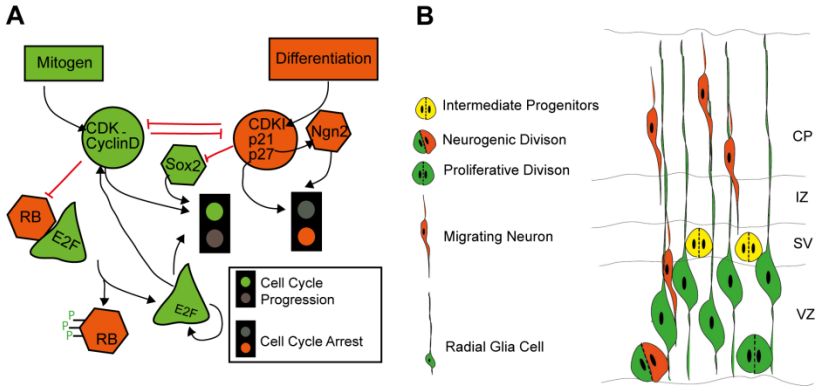

Figure 4. Cell cycle network and schematic representation of the developing cortex.

A) Part of the network that regulates the proliferation/differentiation balance of neural stem cells. Proteins that promote proliferation are depicted in green and differentiation promoting factors in red. B) The cells that compose the cortex are generated outside the cortex and reach their final destination by migration. For example, excitatory neurons are born in the proliferative regions of the developing cortex and reside in the cortical plate. The activity of neural stem cells is tightly regulated to ensure the balanced production of different neuronal subtypes. The two principle classes of neuronal progenitors are radial glia cells and intermediate progenitors which reside in VZ and SVZ respectively. The mitotic activity is induced when the radial glia cells approach and contact the ventricular lumen. This division can lead to generation of neurons which use the scaffold of radial glia cells to migrate and position in the cortical plate.

1.7.1 Cell cycle of neural stem cells

Two major neurogenic niches exist during cortical development; the ventricular zone (VZ) largely populated by the radial glia cells, and the subventricular zone (SVZ), populated by

the intermediate progenitors (Fig. 4B). More recently an additional class of progenitors is described in the outer subventricular zone OSVZ (OSVZ) of human, as well as of ferret and mouse (Fietz, Kelava et al. 2010; Hansen, Lui et al. 2010; Fietz and Huttner 2011). Initially neural stem cells mainly divide symmetrically to expand the pool of neural stem cells (E9.5-E11.5). This phase is followed by gradual increase of neurogenic divisions (E11.5-E17.5) (Dehay and Kennedy 2007; Farkas and Huttner 2008). Neurons can arise directly from radial glial cells or indirectly through intermediate progenitors which undergo symmetric final mitosis to produce two neurons (Dehay and Kennedy 2007; Farkas and Huttner 2008) (Fig. 4B). Before migration starts, the neuronal precursors need to exit the cell cycle. It is noteworthy that progression through the cell cycle and fate specification is highly coordinated (Dehay and Kennedy 2007; Farkas and Huttner 2008). Entry and exit from the cell cycle is regulated during the G1 phase of the cell cycle. Progression through the G1 is tightly regulated by Cyclin Dependent Kinases (CDK2/4/6) whose activity depends on their interaction with their activating cyclins subunits (E/D). When the activity of the G1 CDK-Cyclin is low, retinoblastoma tumour suppressor protein (RB) binds to E2F transcription factors suppressing the transcription of the genes required for cell cycle entry (Fig 4A). In response to mitogen in early G1, CDK4/6-CyclinD Phosphorylates RB resulting in its hyperphosphorylation. Hyperphosphorylated RB is inactive and dissociates from E2F transcription factors leading to the transcription of the genes necessary for transition to S phase of the cell cycle (Figure 4A). Consistent with an anti-proliferative role for RB, telencephalon specific deletion of RB enhances the proliferation of the neural progenitors and an increase in the size of the telencephalon (Ferguson, Vanderluit et al. 2002). Active E2F1 promotes the expression of CDK2/CylinE which triggers the G1-S transition in late stages of the G1. The activity of the CDKs is negatively regulated by CDK inhibitors (CDKIs). There are two classes of the CDKIs: the INK4 family (p15, p16,p18 and p19) and the CIP/KIP (p21, p27, p57). In neural stem cells of the adult brain loss of p21 results in increased proliferation of stem cells followed by their exhaustion. These data show that the cell cycle inhibitor p21 is necessary to maintain the pool of neual stem cells in the adult brain by regulating their quiescence (Kippin, Martens et al. 2005). More recently, it was shown that P21 regulate the pool of neural stem cells by suppressing the expression of Sox2 which is necessary transcription factor for selfrenewal of neural stem cells (Marqués-Torrejón, Porlan et al. 2013). It is interesting that p21 not only regulate the activity of the G1 CDK but also link the cell cycle progression to cell fate decisions mechanisms. p27 is another CDKI which control activity of the CDK2/Cyclin E during G1-S transition. Interestingly, p27 deficiency promotes neuronal differentiation by stabilizing Neurogenin2 (Ngn2) in a cell cycle independent manner (Nguyen, Besson et al. 2006). Together, these findings suggest that cell cycle and

cell fate mechanism are interconnected with some proteins regulating both proliferation and differentiation (Fig 4A).

Early studies of cortical neurogenesis proposed a model in which the length of the cell cycle determines the output of the division. Takahashi et al used S-Phase labelling of dividing neural stem cell to show that the length of the cell cycle increases during cortical development. This increase in the cell cycle length is accompanied by increased rate of neurogenesis meaning that early short cycles expand the pool of neural stem cell, whereas longer cycles in later stages of cortical development produce more neurons (Takahashi, Nowakowski et al. 1995; Takahashi, Nowakowski et al. 1996). Currently, converging evidence suggest that the progressive increase in neurogenesis is mainly due to the lengthening of the G1 phase of the cell cycle. Based on this model, differentiative divisions have longer G1, and the reverse is true for proliferative divisions (Calegari and Huttner 2003; Dehay and Kennedy 2007). Calegari and Huttner used specific inhibitor of CDK2 (Olomoucine) to show that lengthening of G1 progression increases the number of neuron generating divisions (Calegari and Huttner 2003). On the contrary over-expression of CDK4/Cyclin D1 shortens the G1 leading to increased number of proliferative divisions (Lange, Huttner et al. 2009; Pilaz, Patti et al. 2009). Over-expression of cdk4/cyclin D1 did not affect cell size or the cleavage angle of the division, but increased the number of divisions that produce basal progenitors confirming the idea that G1 cdk activity is a major determinant of the differentaitive vs. proliferative division (Lange, Huttner et al. 2009). Similar to developmental neurogenesis, over-expression of cdk4/cyclin D1 in the adult hippocampus expand the pool of adult neural stem cells suggesting conservation of this developmental pathway in adult neurogenesis (Artegiani, Lindemann et al. 2011). Based on these finding, the "cell cycle length" model proposes that the speed of G1 determines the fate of the daughter cells, perhaps by regulating their exposure to extrinsic differentiation factors (Calegari and Huttner 2003; Caviness, Nowakowski et al. 2009).

1.7.2 Radial migration and the role of APP proteins

Post-mitotic neurons born in neurogenic niches migrate towards the cortical plate using the fibers provided by the radial glia cells (Fig. 4B). This mode of migration is called glia guided migration. The glia guided migration stops at a cellular layer populated by Cajal-Retzius (CR) cells. CR cells produce Reelin which binds to ApoER2 and Vldlr receptor to signal via Dab1 to control the end stage of neuronal migration by promoting glia independent somal translocation (Franco, Martinez-Garay et al. 2011). Triple deletion of *App*, *Aplp1* and *Aplp2* result in reduced number of CR cells and accumulation of neurons

that over-migrate to the marginal zones of the developing cortex (Herms, Anliker et al. 2004). Likewise, deficiency of presenilin-1 decreases the number of CR cells and cause an ectopic accumulation of neurons in the marginal zones of the developing cortex (Hartmann, Strooper et al. 1999), suggesting the importance of APP processing in CR cell function. In contrast to the over-migration phenotype, Young-Pearse et al (Young-Pearse, Bai et al. 2007) showed that single knock-down of APP in wt brain inhibits migration of cortical neurons and APP over-expression promoted the migration of neurons which again depends on the conserved YENPTY in the carboxyl terminus. It is noteworthy that endogenous expression of *Aplp1* and *Aplp2* could not rescue the effect of shRNA, but their over-expression rescued the migration phenotypes, indicating that regulated expression of App and Aplps is critical for their function (Young-Pearse, Bai et al. 2007). Down-stream, DISC1 is another interactor of APP with key roles during progenitor proliferation and neuronal migration (Bradshaw and Porteous 2012). Over-expression of DISC1 can significantly rescue the migration effect observed after Dab1 and APP down-regulation (Young-Pearse, Suth et al. 2010). Upstream, binding of different isoforms of Pancortins mediate different effects on processing of APP. Using an unbiased assay for identification of ectodomain binders of APP, Rice et al showed that pancortins can bind to APP and binding of Pancortin 1 and 2 (B-domain containing pancortins) can significantly decrease Abeta processing of APP (Rice, Townsend et al. 2012). Down-regulation of Pancortin 1 or over-expression of Pancortin 4 resulted in similar migration defects observed after down-regulation of APP in developing cortex suggesting opposite roles for different isoforms of the Pancortins (Rice, Townsend et al. 2012). Expression of Pancortin 1 or APP could rescue the delayed migration of pancortin 4 over-expressing migratory neurons (Rice, Townsend et al. 2012). Similar to Pancortins, Reelin interacts with the extracellular domain of APP in primary hippocampal neurons (Hoe, Lee et al. 2009) and Dab1 interacts with the highly conserved YENPTY motif in the carboxyterminus of APP. This interaction most likely depends on the phosphorylation of tyrosine highlighting further the importance of this residue for developmental functions of APP proteins (Howell, Lanier et al. 1999). Extracellular interaction of APP with Reelin and intracellular binding to Dab1 shows that APP might work together with ApoER2/Vldlr as (co)receptor to mediate the Reelin effect during migration of neurons. Indeed, Dab1 over-expression could rescue the blocked migration induced by APP shRNA in the developing cortex, further suggesting that Dab1 might act down-stream of APP and Reelin complex (Young-Pearse, Bai et al. 2007). From these findings, a model emerges in which the ectodomain of APP binds to Reelin and Pancortins at the cell surface which leads to signal transduction through down-stream effectors such as Disc1 and Dab1 to regulate neuronal migration. It is very likely that phosphorylation of YENPTY motif at the C-terminus plays a central role in the regulation

of cortical migration. However, this model does not explain the reduction of CR cell in triple ko mice (Herms, Anliker et al. 2004), unless Reelin affects the survival of CR cells in a cell-autonomous way. It is likely that early neurogenesis is affected by APP proteins because CR cells are among the first neurons that are born during development.

It might be that Reelin has a dual site of action in the developing cortex. Similar to post-mitotic migratory neurons, Reelin-Dab1 can directly signal to radial glial cells regulating their morphology and rate of neurogenesis (Hartfuss, Förster et al. 2003; Lakomá, Garcia-Alonso et al. 2011; Pérez-Martínez, Luque-Río et al. 2012). Regarding the dual site of action of Reelin, it is likely that during cortical development App and Aplp2 can regulate Reelin signalling both in migrating neuron and proliferating neural stem cells. Moreover, the YENPTY motif is also present in App like proteins raising the possibility that Reelin can also signals through Aplps to control migration and differentiation of cortical precursors and progenitors.

Altogether, the current findings do not converge to a concrete model for APP protein family function during cortical development. Moreover, it is not clear to what extent App and Aplps have specialized function in different neuronal events such as migration and differentiation.

Chapter II: Rationale, Aims and Approach

Expression of amyloid precursor protein (APP) and its two paralogues, APLP1 and APLP2 during brain development coincides with key cellular events such as neuronal differentiation and migration. However, the neurodevelopmental roles of APP and APLPs remain controversial. When analyzing the function of APP and APLPs, a primary assumption made by many investigators is that the APP proteins are redundant. However, the early embryonic lethality of triple ko mice precludes the generation of sufficient number of mice for careful analysis of neurons (less than 6.25% theoretical chance of triple ko embryos from viable parents)(Bergmans, Shariati et al. 2010) . Previously, we approached these problems by generation of an embryonic stem cell line with triple deletion of *App/Aplp1/Aplp2*. The triple ko ES cells were differentiated to neurons *in vitro* and were analysed for different parameters such as migration and synaptic activity (Bergmans, Shariati et al. 2010). However, we did not find any obvious phenotype. This prompted us to further investigate the role of the APP gene family *in vivo*. We started by the generation of the chimeric mice with tKO neurons incorporated in a wt background brain using a morula aggregation technique. Using this technique, we showed that triple ko neurons can be generated *in vivo* and can migrate to the cortex (Bergmans, Shariati et al. 2010). However, we could not generate sufficient numbers of mice for in depth analysis of neurons and neuronal precursors *in vivo*.

The enrichment of Aplp2 expression in the proliferative zones of the developing cortex is compatible with a role of this protein in early proliferation and differentiation of neuronal precursors prior to migration. Moreover, among the different combinatorial genetic deletions of App/Aplps in mice, only *App/Aplp1* double ko is a viable genotype, supporting a crucial and distinct developmental role for Aplp2 (Herms, Anliker et al. 2004). Therefore, we down-regulated Aplp2 by shRNA in wt mice as well as in *App/Aplp1* dko mice. We avoided lethality by down-regulating Aplp2 only in a subpopulation of cells in the ventral telencephalon. This was done by *in utero* electroporation of shRNA against Aplp2. We investigated the effect of the loss of Aplp2 function in the proliferation and differentiation of progenitors, and migration and final positioning of cortical excitatory neurons using specific promotors to drive cell specific expression of the shRNA.

Chapter III: Methods and Materials

The following chapters partly correspond to the following research article:
S. Ali M. Shariati et al, APLP2 regulates neuronal stem cell differentiation during cortical development, Journal of Cell Science, 126, 2013.

3.1 DNA constructs

We used two different approaches to down-regulate expression of the APLP2. First, we have used a type III RNA polymerase promoter (U6) to express APLP2 shRNA. The hairpin itself is composed of the 29nt followed by 7nt loop structure and 29nt reverse complement sequence. Trancription of shRNA by U6 promoter produces shRNAs that are recognized by miRNA processing enzyme to generate targeting sequence (Rossi 2008). In the second approach, the shRNA is flanked by the genomic sequence of a naturally present miRNA, in this case Let7f2. The let7f2 based shRNA is expressed from an intron of the GFP allowing cell specific expression by using RNA polymerase II expression (Chang, Elledge et al. 2006).

The APLP2 shRNA1 (GI562807) (sequence: 5'-CGATTACAATGAGGAGAATCCAACCGAAC-3'), the APLP2 shRNA2 (GI562808) (Sequence 5'-ATGAAGGCTCTGGAATGGCAGAACAAGAC-3') and control shRNA (scrambled sequence: 5'-GCACTACCAGAGCT AACTCAGATAGTACT-3') driven by the U6 promoter were obtained from Origene (Rockville, USA).

The *pCAG-EGFPintron-let-7f* based shRNA expression system was constructed as followed. The synthetic intron found in the psicheck2 plasmid (Promega, Leiden, The Netherlands) was PCR amplified using Promega IntronF (5'-CGAAGGTAAGTATCAAGGTTACAAGACAG-3') and R (5'-GACGTAGCCTGTGGAGAGAAAGGCAAAGTG-3') primers. The intron was then inserted into EGFP by overlap-PCR using two inner primers for 5' (5'-TGATACTTACCTTCG GGCATGGCGGACTTGAAG-3') and 3'arms (5'-TCTCTCCACAGGCTACGTCCAGG AGCGCACCATCTTCTTC-3') of EGFP and two outer primers for 5' (5'-GCCACCGGTCGATCCACGCCACCATGGTGAGCAAGGGCG AGGAG-3') and 3' (5'-GATTGTCGACTTACTTGTACAGCTCGTCCATGCCG-3') arms. Next, XhoI and EcoRI restriction sites were added to the intron by PCR using Intron XhoI-EcoRI-F (5'-GAATTCCAATCTCGAGCTATTGGTCTTACTGACATCCACTTTGC-3') and Intron XhoI-EcoRI-R (5'-CTCGAGATTGGAATTCAGCCTATCAGAAACGCAAGAG TCTTCTCTG-3') primers (pCAG-EGFP intron). The let-7f2 genomic sequence was

amplified from human genomic DNA using LET-7Fhu-MfeI (5'-TCATCAATTGTAACTCTCCTTCCCTTTCTCCCTTCTTAC-3') and LET-7Fhu-SalI (5'-TCATGTCGACCATCAAAGGACCAGCCACTT-3') primers and cloned into the pCAG-EGFP intron vector digested by XhoI and EcoRI. This intermediate construct contains the genomic sequence of human let-7f2 precursor including the mature let-7f sequence. In order to remove this mature sequence and facilitate cloning of shRNAs, the 5' and 3' arms of the let-7f scaffold were amplified using two inner primers: 5'arm (5'-GGCGCGCCCTCGAGCCATCTTCAGCCTATGTGGG-3') and 3'arm (5'-GGCGCGCCG AATTCTCTTCTCCGACTGGCTCTGTTC-3') scaffold and two outer primers: Let7F-XhoI (5'-CAATCTCGAGGTGCTCTGTGGGAT-3') and Let7F-EcoRI (5'-CAATGAATTCGT ACCACCGTGGGA-3'). The PCR product was cloned into the intermediate construct resulting in the pCAG-EGFPintron-let-7f plasmid. For shRNA cloning, overlapping DNA oligonucleotides were designed to embed the shRNA into the let-7f scaffold sequence. The shRNAs for APLP2 and mCherry were obtained after annealing the following oligonucleotides: APLP2-let7-I (5'-CTCGAGGTGCTCTGTGGGATCGCTGCTGGGT TCGGTTGGATTTAGGGTCATACCCCATCTTG-3'); APLP2-let7-II (5'-GAATTCGTAC CACCGTGGGACGCCACTGGGTTCGGTTGGATATCTCCAAGATGGGGTATGAC-3'); mCherry-let7-I (5'-CTCGAGGTGCTCTGTGGGATGATGTTGACGTTGTAGGCGCCTT AGGGTCATACCCCATCTTG-3'); mCherry-Let7-II (5'-GAATTCGTACCACCG TGGGAGATACTGACGTTGTAGGCGCCATCTCCAAGATGGGGTATGAC-3') and PCR amplified using pre-Let7F and pre-Let7R universal primers. The resulting shRNAs were digested and cloned into the pCAG-EGFPintron-let-7f plasmid using XhoI and EcoRI restriction sites.

BLBP-shRNA mir: For cell-specific expression into radial glia cells, the BLBP promoter was amplified from mouse genomic DNA using BLBP-F (5'-CAATGTCGACAG CACAGCAGAAAGGGAAAA-3') and BLBP-R (5'-GGTGGGCGCGCCAGGCAGGAACT GGAGGAACTC-3') primers and cloned into the pCAG-EGFPintron-let-7f digested by SalI and AscI, thus replacing the CAG promoter by the mouse BLBP promoter.

Tα-shRNAmir: The tubulin alpha promoter was chosen to drive neuronal expression and amplified from mouse genomic DNA using Tα-F (5'-ACCTACTAGTGTATTAGAA GGGATGGCTCA-3') and Tα-R (5'-ACCTACCGGTGGTTGCTGCTTCGCGGCTGCC-3') primers and cloned into the pCAG-EGFPintron-let-7f digested by SpeI and AscI. For *in*

utero electroporation, DNA preparations, included endotoxin removal treatment, were obtained using Qiagen EndoFree Plasmid Maxi Kit (Qiagen, Venlo, Nethelands), with final concentration between 2-3 µg/µl plasmid DNA.

3.2 Western blot

Total cell lysates of cortical neuron cultures or HEK293 were prepared in cell lysate buffer (1 % Triton-X100, with protease inhibitors in PBS). 20 µg of protein was separated on a NuPAGE 4 %-12 % (Invitrogen), transferred to nitrocellulose and membranes were incubated overnight at 4 °C with the following primary antibodies: APLP2 (CT12 a kind gift from Dr. Thinakaran), V5 antibody (1:10000-mouse, Invitrogen Gent, Belgium), APP antibody (B63-1:5000, custom made rabbit antibody), GAPDH (1:5000-mouse, HyTest), Actin (1:1000-mouse, Sigma-Aldrich, Diegem, Belgium) and detected with HRP conjugated secondary antibodies using a ECL chemiluminescence detection kit (PerkinElmer Life Sciences, Zaventem, Belgium). The density of bands was quantified by densitometry using Aida Image Analyser 4.27 (Raytest, Straubenhardt, Germany) and linearity of the signal was tested using different dilution of total cell lysate.

3.3 Cell cultures

Mouse embryonic cortical neurons were prepared as described previously (Banker & Goslin, 1988) and plated at a density of 100000 cells per cm^2 on poly-L-lysine (PLL) coated dishes. Neurons were transfected before plating using nucleofection (Amaxa, Cologne, Germany). HEK293 cells over-expressing V5 tagged APLP2 were grown in DMEM/F12 with 10 % Fetal Calf serum (FCS) and were transfected with shRNA expressing construct using TransIT®-LT1 transfection reagent (Mirus, Madison, USA).

3.4 Matrigel assay for migration

E14 embryonic cortices were dissected and digested by papain for 20 min at 37 °C. After subsequent mechanical dissociation, cells were transfected by nucleofection (Amaxa) followed by overnight shaking (350rpm) at 37°C to form aggregates. The aggregates were embedded in Matrigel (BD Biosciences, Erembodegem, Belgium) on coverslips and fixed in 4 % paraformaldehyde (PFA) after 3 to 4 days. For live imaging from one day after plating onwards, coverslips were mounted in a closed metal chamber and images were acquired at 20 min intervals for up to 24 h using an inverted Olympus Cell^R microscope.

3.5 Knock-out mice

APP/APLP1 double knock-out mice were described previously and generated by genomic deletion of the promoter and initiation codon of APP and APLP1 loci (Heber, Herms et al. 2000; Herms, Anliker et al. 2004). Wt embryos were from C57/Bl6 background.

3.6 In utero electroporation

All animal experiments were approved by the Ethics Committee of the K.U.Leuven. Pregnant mice were anesthetized by intramuscular injections of 88 µg ketamine and 132 µg xylazine per gram of body weight. The uterine horns were exposed and the plasmids (1-2 µg/µl) mixed with Fast Green (Sigma) were microinjected in the lateral ventricles of E14.5 mouse embryos. Five current pulses (50 ms pulse/ 950 ms interval) were delivered across the head of the embryos (36 V) targeting the dorsal-medial part of the cortex. After 2-4 days, embryos were collected and perfused with PBS and 4 % PFA and the brains postfixed for 6-10 h in 4 % PFA at 4 °C.

3.7 Immunocytochemistry

Coronal vibratome sections of the fixed embryonic brains were prepared with 100 µm thickness. Subsequently, the sections were permeabilized and blocked at RT for 1 h in PBS/ 0.3 % Triton-X100/ 3 % BSA/ 5 % goat or donkey normal serum, incubated with the primary antibody at 4 °C overnight followed by the secondary Alexa conjugated antibodies for 2 h at room temperature (Invitrogen). For BrdU detection, slices were pre-treated with 1M HCl (10 min 4 °C) and 2 M HCl (10 min RT and 20 min 37 °C) with subsequent washes in 0.1 M borate buffer.

The following primary antibodies were used: chicken anti-EGFP (1:500; Aves labs, Oregon, USA), rabbit anti-Ki67 (1:300; Novacastra, Diegem, Belgium), rabbit anti-Tbr2 (1:1000; Abcam, Cambridge, UK), rabbit anti-PH3 (1:300; Cell signalling, Leiden, The Netherlands), rabbit anti-βIII-tubulin (1:1000, Abcam), mouse anti-BrdU (1:200; Roche, Vilvoorde, Belgium), rabbit anti-Cux1 (1:500; Santa-Cruz, Heidelberg, Germany), goat anti-Sox2 (1:150; Santa-Cruz). Nuclei were visualized with DAPI.

3.8 Cell cycle exit

One day after *in utero* electroporation, pregnant mice were injected intraperitoneally with BrdU (75 mg/kg, Sigma-Aldrich). After another 24h the brains were collected, fixed and immuno-stained using anti-EGFP, anti-BrdU and anti-Ki67 antibodies. The cell cycle exit rate was calculated as the ratio of $EGFP^+/BrdU^+/Ki67^-$ cells (cells which are not in the cell cycle) divided by the number of $EGFP^+/BrdU^+$ cells (total number of dividing and non-dividing cells).

3.9 Confocal imaging and quantification

Confocal images were captured on a Nikon microscope (Eclipse; Ti A1) using an Apo 10× A/1.40 N.A. objective lens. The images were acquired by Nis-Element software and the imaging parameters were kept constant during imaging. Ten to fifteen consecutive Z-sections were obtained per brain slice. All images were processed using the ImageJ software (NIH).

For cortical positioning: The entire length of cortical walls was divided into ten equal bins and the frequency of cells per bin was calculated by counting the cell bodies of EGFP-Positive cells in each bin, divided by the total number of EGFP positive cells.

For cell cycle exit: All the images were thresholded and BrdU+/EGFP+ cells were detected by the AND function of Image Calculator (ImageJ software). Next, the same function was used to find BrdU/EGFP double positive cells that are positive or negative for Ki67.

3.10 Statistics

Corresponding bins were compared using Student's *t*-test. The population distribution of two groups of neurons was compared using a non-parametric Mann–Whitney U test ($P < 0.05$ as significance level). All statistical analysis and graph preparation were done by using GraphPad Prism5.

Chapter IV: Results

To study the role of Aplp2 in neuronal development, we tested the effect of Aplp2 down-regulation on cortical position of neurons. To this end, we performed the following experiments:

- Identification of functional and specific shRNAs
- Down-regulation of Aplp2 in wt cortices using *in utero* electroporation
- Down-regulation of Aplp2 in dko cortices using *in utero* electroporation
- Morphological studies of neurons and neuronal precursors
- Cell specific interference with Aplp2 functions
- Analysis of cell cycle variables

In these experiments, we electroporated Aplp2 shRNA at E14 and removed the brain at E18 for migration and morphological studies. The neurons born on E14 are expected to reside in the upper layers of the developing cortical plate 4 days after electroporation (E18.5) (Figure 5).

To analyze the cell cycle, differentiation and mitosis, we collected samples at E16.5 when many electroporated cells are still residing in the VZ/SVZ (Figure 5).

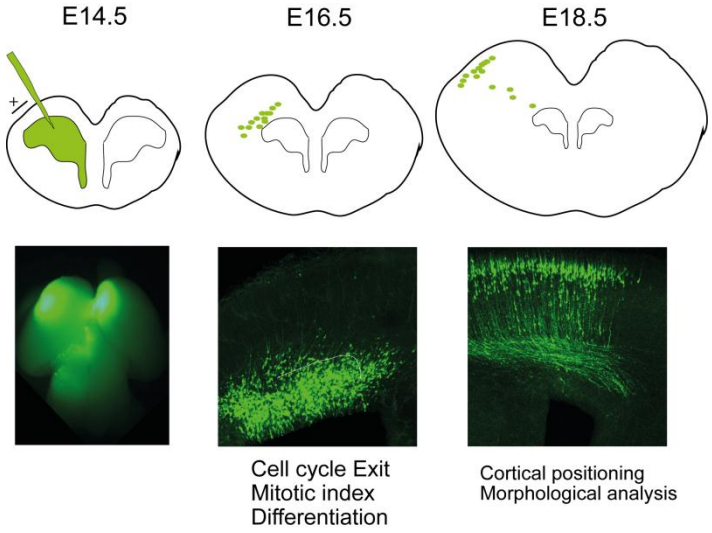

Figure 5. The experimental approaches used to study different neuronal processes.

The DNA constructs expressing Aplp2 shRNA were electroporated at E14.5 Two days after electroporation, most of the cells are still residing in the proliferative zones of the developing cortex. We chose E16.5 (when many of the electroporated cells are still in the proliferative zones) to test the cell cycle exit, mitotic index and differentiation of neuronal precursor. Four days after electroporation, most of the targeted cells end up in the upper layers of the developing cortex. We performed the migration, morphological studies and cortical positioning analysis at E18.5.

4.1 Identification of functional and specific shRNAs targeting Aplp2

To examine the role of Aplp2 in cortical development, we tested the effect of four U6 driven shRNAs on the expression of endogenous Aplp2 in mouse embryonic fibroblasts. We selected two different U6 driven shRNAs constructs (shRNA1 and 2) targeting different regions of the coding sequence of the Aplp2 transcript. To control the effect, we used a scrambled control shRNA construct that does not align significantly with any published NCBI mouse transcript sequence. Western-blot analysis of Aplp2-V5 over-expressing HEK293 cells which were transfected with one of those shRNA constructs confirmed the down-regulation of Aplp2 protein (APLP2 shRNA1 more than 95 % knock-down and Aplp2 shRNA 2 more than 80 % knock-down: Fig. 6A). Since Aplp2 shRNA1 was more efficient for the down-regulation of Aplp2 protein, we used in most of our analysis this shRNA construct (in the further text named Aplp2 shRNA) and utilized the

second construct in initial experiments in order to validate the phenotype and exclude the possibility of off-target effects. Also in cultures of cortical neurons from E14 mice (Fig. 13D) endogenous Aplp2 expression was clearly down-regulated by Aplp2 shRNA1 three days after transfection. Next, we tested the selectivity of the Aplp2 shRNAs by assessing their effect on highly related Aplp1 transcripts. Western blot analysis showed that Aplp2 shRNAs do not change significantly the level of Aplp1 supporting the specificity of selected shRNAs (Fig. 6B).

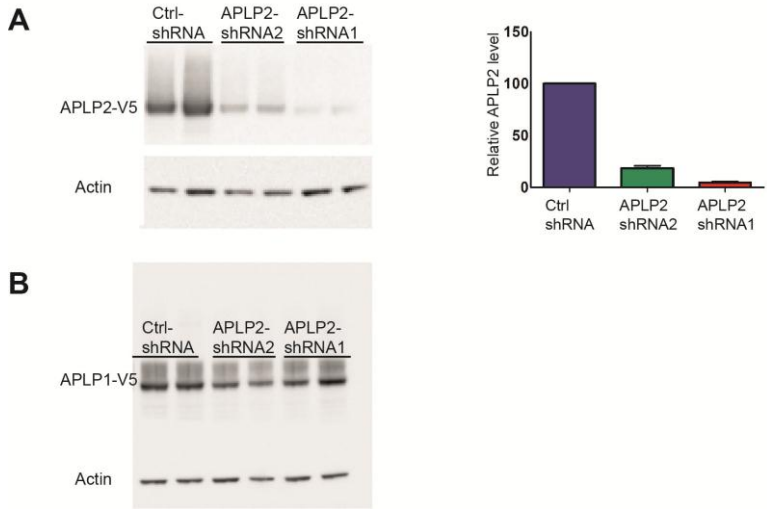

Figure 6. Western blot analysis of Aplp2 shRNA effect

A) The selected shRNA1 and shRNA2 significantly down-regulate expression of Aplp2 –V5 that is overexpressed in Hek293 cells. On the left, the expression of Aplp2-V5 was assessed using a mouse antibody against the V5 tag and on the right the quantification of the APLP2 band normalized to actin loading control (n=2) is shown B) APLP2 shRNAs does not change significantly Aplp1 level. The expression of Aplp1-V5 was assessed using a mouse antibody against the V5 tag.

4.2 Down-regulation of Aplp2 in wt cortices does not change cortical positioning

To address Aplp2 function, we expressed the Aplp2 shRNA constructs in cortical progenitors of wt mice at E14.5 by using *in utero* electroporation. Aplp2 is highly expressed in the developing cortex at E14.5 (Lorent, Overbergh et al. 1995; López-

Sánchez, Müller et al. 2005) when upper layer neurons are generated (Molyneaux, Arlotta et al. 2007). In order to visualize electroporated cells, EGFP was co-expressed. As control, we used a scrambled control shRNA that does not align significantly with any NCBI mouse transcript sequence. Four days after electroporation, the time point when transfected precursors are expected to have differentiated into neurons and are residing in the upper layers of the cortical plate (Fig. 7A), we fixed the brains and analysed the position of labelled cells in coronal sections. For this purpose we divided the cortex into 10 equal bins and counted the relative number of EGFP-positive cells in each bin (Fig. 7A/B, see Material and Methods). Moreover, we analysed the difference of the entire population distribution (Fig. 7B, inset). Both types of analysis did not reveal any difference in the behaviour of Aplp2 shRNA expressing cells in respect to control shRNA cells (Fig. 7A/B). In both cases, EGFP-positive cells were mostly positioned in the upper layers of the cortex, which is marked by Cux1 (Cut like homeobox 1)antibody (Fig. 7B).

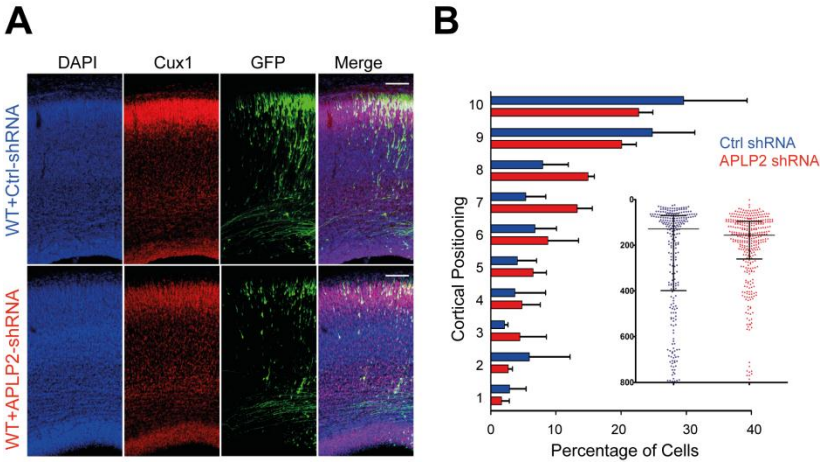

Figure 7. APLP2 down-regulation in wt cortices does not change cortical positioning of neurons.

A) Confocal images of coronal slices of wt brains electroporated with constructs expressing Aplp2 shRNA or Ctrl shRNA together with GFP, 4 days after electroporation (E14.5-E18.5). Aplp2 shRNA expression does not lead to developmental differences respect ctrl conditions. B) Quantification of GFP+ cells of A. Bar graphs represent frequency distribution of GFP positive cells in ten equally divided bins from ventricle (10) to the pial surface (1) of the cortical wall. Values represent the mean ± STDV (n = 5; t-test). The inset scatter plot compares the population distribution of GFP+ cells. (n=3, 300-400 cells only are shown for clarity of graph; Values represent the median ± interquartile Mann Whitney test).

4.3 Down-regulation of Aplp2 in *App/Aplp1* dko cortices affects cortical positioning

Next, we considered the possibility that the overlapping function of App, Aplp1 and Aplp2 throughout development (Heber, Herms et al. 2000) could have led to a compensation for the loss of Aplp2 function. To test this, we expressed Aplp2 shRNA in cortical progenitors from App/Aplp1 dko mice. Aplp2 down-regulation in *App/Aplp1* dko resulted in a large number of EGFP positive-cells (54 %) residing in the Cux1 negative region, i.e. predominantly in the VZ/SVZ of the developing cortex (Fig. 8A/B). Similar to wt mice, the majority of neurons electroporated with the control shRNA construct (*App/Aplp1* dko) migrated to the upper layer of the cortical plate (Cux1-positive layer, Fig. 8A). This indicates that Aplp2 is an important component of the machinery responsible for proper neuronal progression towards the cortical plate. To guard against possible off-target of the Aplp2 shRNA construct, we used a second shRNA (i.e. shRNA2) to target Aplp2 in the developing cortex. Again, we find only a change in the cortical positioning of cells transfected with Aplp2 shRNA2 in *App/Aplp1* dko mice (Fig. 9 A,B). Moreover, the inability of both Aplp2 shRNA constructs to induce any phenotype in wt cortices (Fig. 7A,B and Fig. 9A,B) argues for the specificity of our approach.

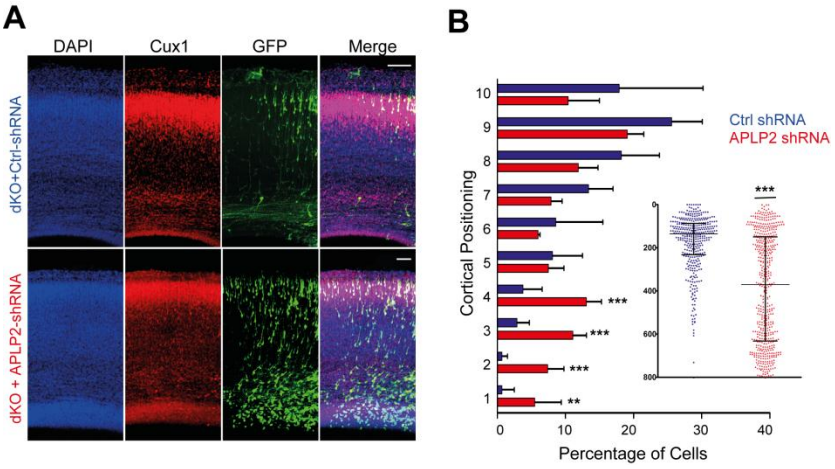

Figure 8. APLP2 down-regulation in dko cortices change cortical positioning of neurons.

A) Confocal images of coronal slices of dko brains electroporated with constructs expressing Aplp2 shRNA or Ctrl shRNA together with GFP, 4 days after electroporation (E14.5-E18.5). Aplp2 shRNA expression leads to developmental differences respect wt and ctrl conditions. B) Quantification of GFP+ cells of A. Bar graphs represent frequency distribution of GFP positive cells in ten equally divided bins from ventricle (10) to the pial surface (1) of the cortical wall. Values represent the mean ± STDV (n = 5; t-test). The inset scatter plot compares the population distribution of GFP+ cells. (n=3, 300-400 cells only are shown for clarity of graph; Values represent the median ± interquartile Mann Whitney test).

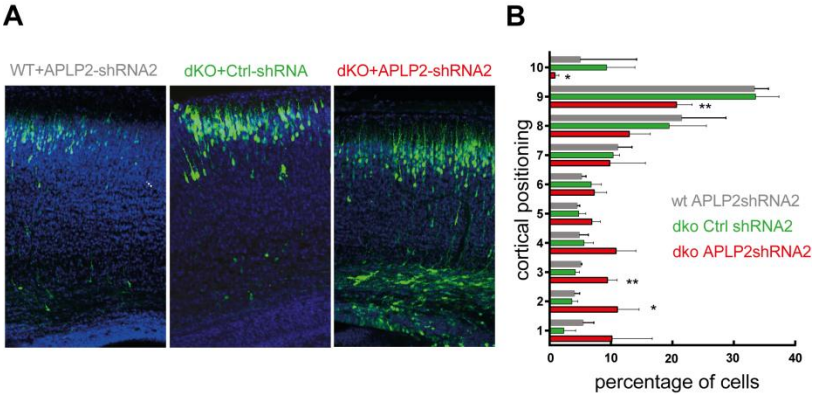

Figure 9. Confocal images (left) and quantification (right) of the effect of a second Aplp2 shRNA.

See the text and legend of figure 7 and 8 for full description.

4.4 Morphology and migratory behaviour of "Triple knock-out" cells remain unchanged

Next, we asked whether the morphology of neurons that are moving towards the cortical plate is different in dko vs. "triple ko" neurons ("triple ko" refers to APP/APLP1dko cells expressing APLP2shRNA). Neurons born in the proliferative zones of the cortex acquire a multipolar morphology in the lower part of the intermediate zone. As they migrate towards cortical plate, the neurons change from multipolar to bipolar morphology with a thick leading process and a thin trailing process. The transition from multipolar to bipolar morphology is essential for proper positioning of the neurons in the cortical plate. A defect in acquiring the bipolar morphology could explain the observed changes in cortical positioning of the cells (Jossin and Cooper 2011). Careful microscopic analysis revealed that this is not the case: neurons in both groups displayed the typical bipolar morphology of migrating neurons with a thickened leading edge and a thinner trailing process (Fig. 10A), indicating that Aplp2 is not essential for the acquisition of the morphological polarization required for proper migration. To assess directly neuronal migration, we monitored the migration speed and distance of dko neurons either expressing the control shRNA or the Aplp2 shRNA through the use of an *in vitro* migration assay in Matrigel (Calderon de Anda, Gartner et al. 2008). We did not detect any differences in the distance that double or "triple ko neurons" migrate away from the explants (Fig. 2B). Moreover, live imaging of migrating neurons did not reveal differences in the speed of neuronal migration nor in the morphology nor in the behaviour of the migrating neurons (Fig. 10B). Thus, neither a morphological defect nor the migratory behaviour of neurons is the cause of altered cortical positioning.

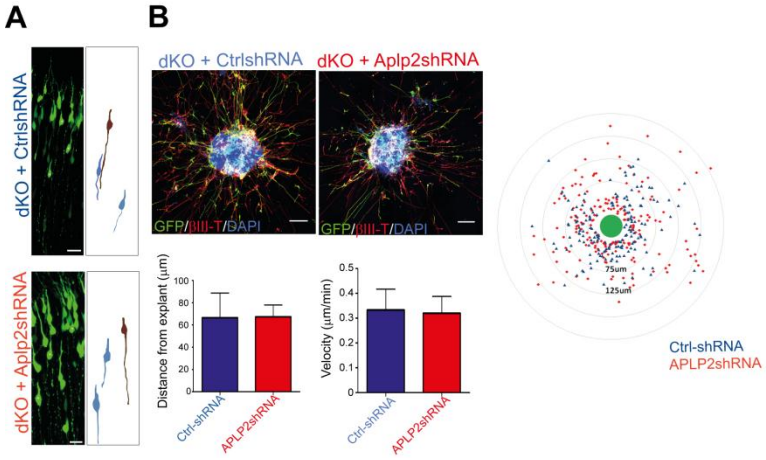

Figure 10. Morphology and *in vitro* migration of migrating neurons are not altered

A) Confocal images of 20-25 (0.8 µm) consecutive z-sections illustrating morphology of dko migrating neurons transfected with Aplp2 shRNA or control shRNA. Drawings on the right depict the morphology of two migrating neurons (blue) and one positioned in the CP. The morphology of neurons in both groups is comparable. Scale bars: 25 µm. B) Images of control shRNA or Aplp2 shRNA transfected neuronal explants embedded in Matrigel 3 days after plating. The first upper graph shows the mean distance of EGFP+ cells from the margin of explants which does not change in "triple ko" neurons. Values represent the mean ± s.d. (n=3 different experiments, Control: 161 cells from 16 explants; shRNA: 182 cells from 18 explants, Student's *t*-test). The second upper graph, on the right, shows the overall distribution of neurons in both groups in respect to the margins of the aggregates. The lower graph shows that the velocity of neuronal movement does not change with expression of Aplp2 shRNA (n=2 independent experiments, 29 cells for Aplp2 shRNA and 26 cells for control shRNA). Scale bars: 50µm.

To test whether the migration delay was due to a change in the morphological differentiation of radial glia cells, which provide the scaffold for radially migrating neurons, we analysed the morphology of EGFP-labelled radial glia cells in E16.5 dko slices of mice with down-regulated Aplp2 from E14.5. The morphology of radial glia was similar in Aplp2 shRNA and control plasmid electroporated dko cortices: they were radially oriented, well aligned and spanned the entire cortical wall with branched basal end feet and apical connections (Fig. 11) (Chanas-Sacre, Rogister et al. 2000).

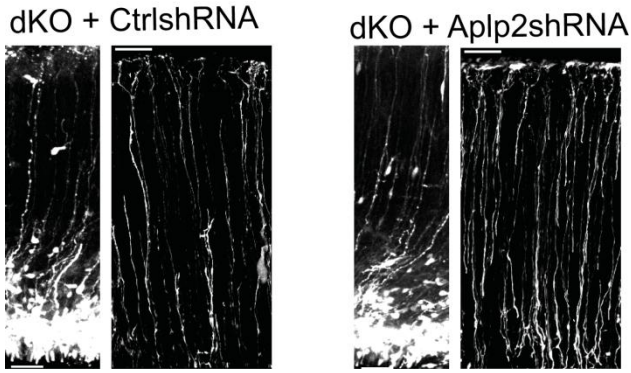

Figure 11.The morphology of radial glia cells remains unchanged.

Confocal projection images of 20-25 consecutive z-sections (0.8 µm) of radial glia cells illustrating their ascending fibers (left) and end feet (right) in *App/Aplp1* double knock-out cortices transfected with Aplp2 shRNA or Control-shRNA. In both groups the fibers span the entire cortical wall with branched end feet that are attached to the pial surface. Scale bars: 50µm (left), 25µm (right).

4.5 "Triple knock-out" cells remain in a proliferative progenitor state

Apart from the defective cortical positioning of "triple knock-out" neurons described above, we also detected retention of "triple knock-out" cells in the VZ/SVZ (Fig. 8A,B). To determine whether the cells arrested in the VZ/SVZ are progenitors which remain longer in a proliferative state, or neurons which fail to migrate away from the proliferative zones, we labelled the brain slices with the basal progenitor marker Tbr2 (T box brain protein 2) (Fig. 12A). In dko cortices electroporated with the control shRNA a small number of EGFP positive cells were located in the VZ/SVZ and only 7 % (± s.d. 2,4 %, n=3) were Tbr2 positive, demonstrating that four days after electroporation, most of the cells became post-mitotic and migrated to the cortical plate (Fig. 12A). In contrast, in dko cortices electroporated with the Aplp2 shRNA, a large number of cells remained in the VZ/SVZ of which 29 % (± s.d. 3.5 %, n=3) expressed Tbr2 suggesting that they failed to differentiate into post-mitotic neurons (Fig. 12A). In addition, a Tbr2 negative population in the ventricular zone, most likely radial glia progenitors (Fig. 19), was still present (Fig. 12A,B) and some cells (Fig. 12B) were still expressing phosphor-histone 3 (PH3), a marker tightly associated to chromosome condensation during mitosis (Goto, Tomono et al. 1999), showing that they were undergoing mitosis. In contrast, in dko cortices electroporated with

the control shRNA, we very rarely found mitotic PH3/EGFP-positive cells and only observed a very small number of radial glia cell progenitors.

Altogether, these data suggest that Aplp2 plays a role in the normal progression of the neuronal differentiation program from precursors to post-mitotic neurons.

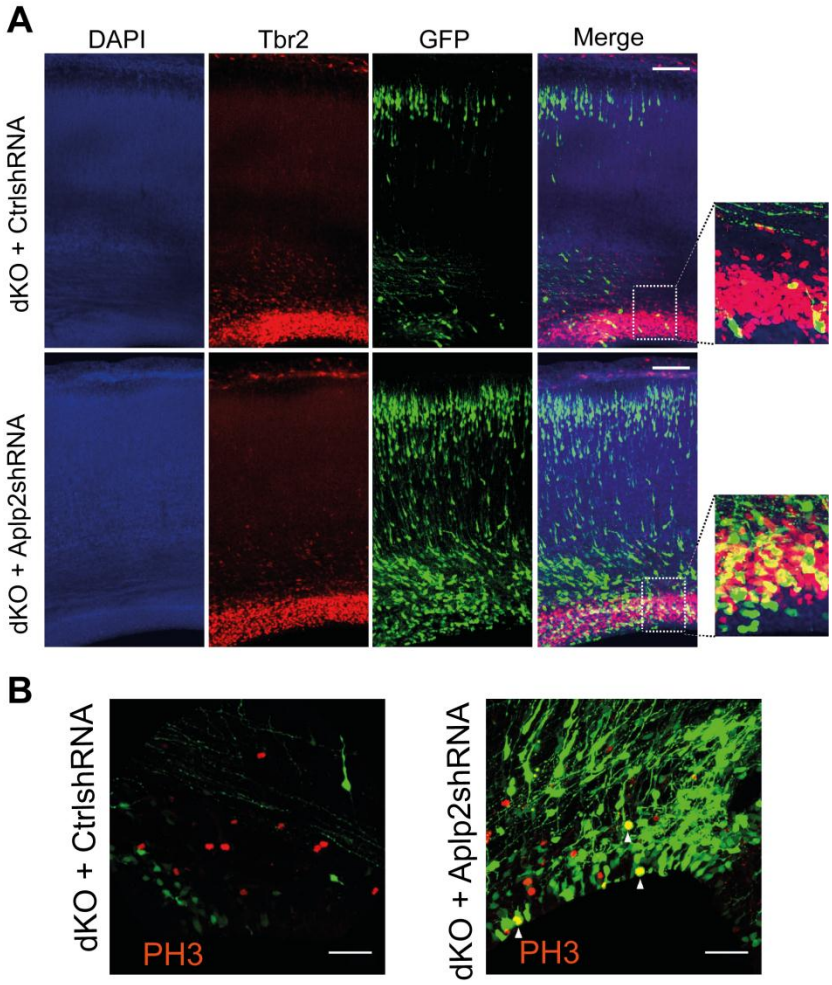

Figure 12. The arrested ‘’triple ko’’ EGFP positive cells express progenitor and mitotic marker.

A) Confocal images of coronal cortical sections labelled with Tbr2 antibody four days after electroporation show progenitors in the VZ/SVZ of Aplp2 shRNA treated cortices. Scale bars: 100 µm. B) *App/Aplp1* double knock-out cortices transfected with Aplp2 shRNA or control shRNA labelled with the mitotic PH3 marker show mitotic Aplp2 shRNA transfected cells (filled arrows). Scale bars: 50 µm.

4.6 Aplp2 has a progenitor specific function and is dispensable for radial migration

To substantiate the above hypothesis, we designed a construct to achieve cell-specific expression of the Aplp2 shRNA, based on the use of a let-7 microRNA embedded shRNA that can be expressed from cell-type specific promoters (Chang, Elledge et al. 2006) (Fig. 13A). In our hand, the let-7 microRNA based shRNA was more effective in down-regulation of cherry fluorescent molecule compared to the commonly used miR-30 microRNA system in Hek293 cells (Fig. 13B). Its functionality for Aplp2 down-regulation was shown by western blot analysis of HEK293 cells expressing a V5 tagged Aplp2 cDNA and transfected with a shRNAmir construct (Fig. 13C). Similar to the U6-Aplp2 shRNA constructs (Fig. 6A), the Aplp2 shRNAmir construct reduced Aplp2 protein expression by about 90 %. Moreover, E14 cortical cultures transfected with Aplp2 shRNAmir, showed comparable down-regulation of endogenous Aplp2 to the U6-driven-shRNA (Fig. 13D). In addition, *in utero* electroporation of Aplp2 shRNAmir using the ubiquitous CAG promoter recapitulated the phenotype (14A) that was obtained by the U6 driven Aplp2 down-regulation in dko mice (Fig. 8 A,B).

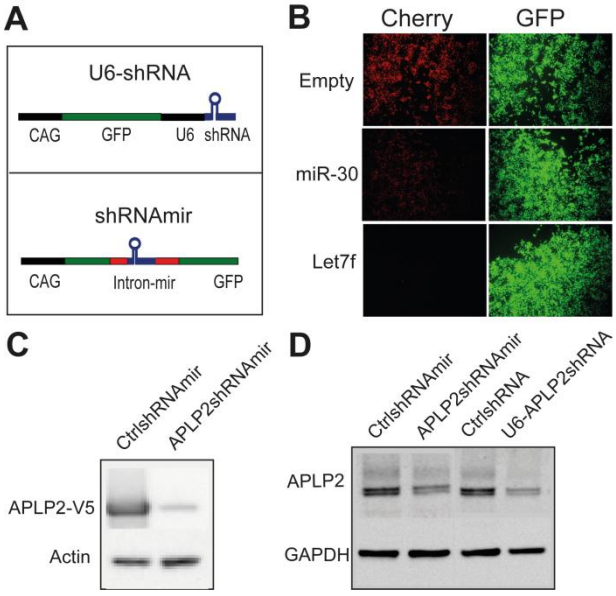

Figure 13. Cell specific construct for Aplp2 down-regulation

A) Schematic comparison of U6-shRNA and microRNA-based shRNA. U6 is a type III RNA polymerase promoter that is expressed ubiquitously, whereas microRNA based shRNA can be expressed from cell specific type II RNA polymerase promoters B) Down-regulation of the

cherry fluorescent molecule using miR30 or Let7f2 based shRNA GFP construct in Hek293 cells. Let7f2 construct appeared more powerful in down-regulating Cherry when compared to miR30 system. C) Western blot showing protein levels of Aplp2-V5 and actin (loading control) of HEK cells expressing Aplp2-V5 transfected with Aplp2 shRNAmir or control shRNAmir. The down-regulation obtained by the microRNA construct was about 90 % and comparable to the efficiency of the U6 shRNA1 construct (see Fig. 6A) D) Aplp2 and GAPDH (loading control) western blot of cortical cells transfected by shRNAmir or U6 shRNA directed against Aplp2. Comparable down-regulation was obtained by both constructs.

This system allowed us to investigate the result of a cell specific loss of Aplp2 function by driving the expression of Aplp2 shRNA in neural progenitors and post-mitotic neurons using the Brain lipid-binding protein (BLBP) and Tubulin-α (Tα) promoters respectively (Feng, Hatten et al. 1994; Gloster, Wu et al. 1994; Coksaygan, Magnus et al. 2006; Hashimoto-Torii, Torii et al. 2008). Differential expression of Tα and BLBP promoters was confirmed by co-electroporation of Tα-mCherry with BLBP-EGFP *in utero* (Fig. 14B).

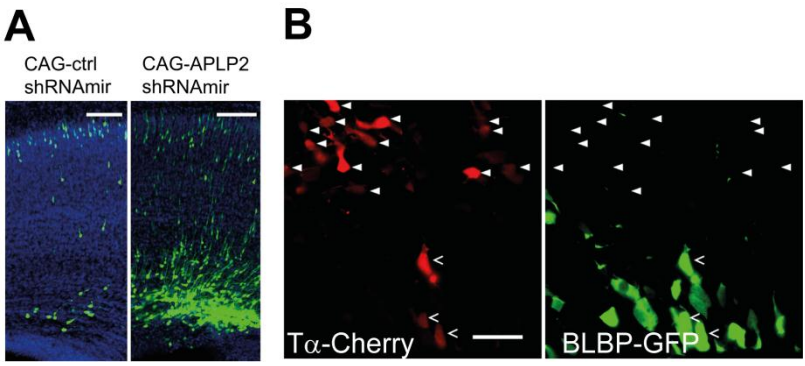

Figure 14. Down-regulation of Aplp2 by shRNAmir change the cortical positioning

A) Confocal images of coronal slices of dko brains electroporated with constructs expressing Aplp2 shRNAmir or Ctrl shRNA from Let7 construct, 4 days after electroporation (E14.5-E18.5). Aplp2 shRNAmir expression lead to the similar developmental defect as U6 driven shRNA. B) Confocal images (projection of 10-15 consecutive z-sections) of dko cortical slices transfected with Aplp2 shRNAmir or control shRNAmir under the control of Tα promoter. Neuronal down-regulation of Aplp2 does not change cortical positioning. Scale bars: 100µm.

Expression of Aplp2 shRNAmir under the control of neuronal Tα promoter in dko post-mitotic cells did not result in any changes in the cortical positioning of EGFP-positive cells when compared with control shRNA (Fig. 15A,B).

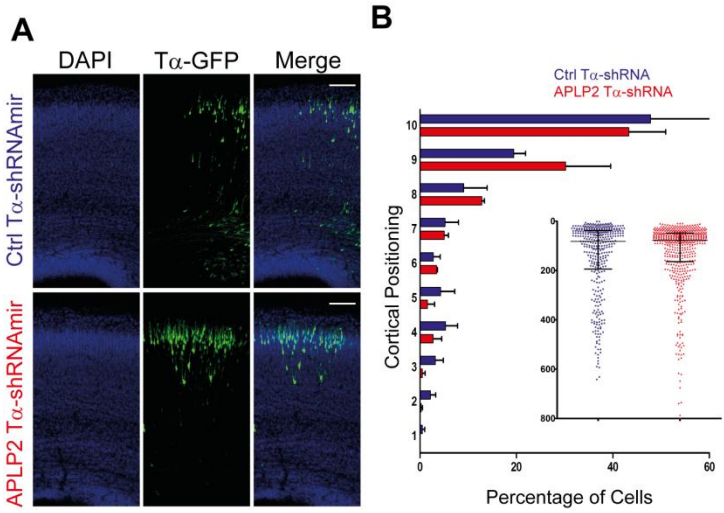

Figure 15. Neuronal Aplp2 shRNAmir does not alter cortical positioning

A) Confocal images (projection of 10-15 consecutive z-sections) of dko cortical slices transfected with Aplp2 shRNAmir or control shRNAmir under the control of Tα promoter. Neuronal down-regulation of Aplp2 does not change cortical positioning. Scale bars: 100µm. B) Quantification of EGFP+ cells of D. Bar graphs represent frequency distribution of EGFP positive cells in ten equally divided bins from ventricle (1) to the pial surface (10) of the cortical wall. Values represent the mean ± s.d. (n=3; Student's *t*-test). The inset scatter plot compares the population distribution of EGFP+ cells. (n=3, 300-400 cells for clarity of graph; Values represent the median ± interquartile range, Mann Whitney test).

In contrast, progenitor specific expression of Aplp2 shRNAmir caused the accumulation of cells in the VZ/SVZ similar to the phenotype that we observed using the U6 promoter (Fig. 16A,B compare to Fig. 8A/B). In order to visualize the progeny of cells electroporated with BLBP Aplp2 shRNAmir after the BLBP promoter is switched off in young neurons, we co-electroporated a mCherry expressing construct with the ubiquitous CAG promoter. Four days after electroporation we detected BLBP-EGFP positive cells only in the Aplp2 shRNAmir expressing cortices (Fig. 16A). This supports a progenitor specific function of Aplp2 and the delay in the exit from the progenitor stage, further highlighting the importance of Aplp2 in neural differentiation of cortical progenitors.

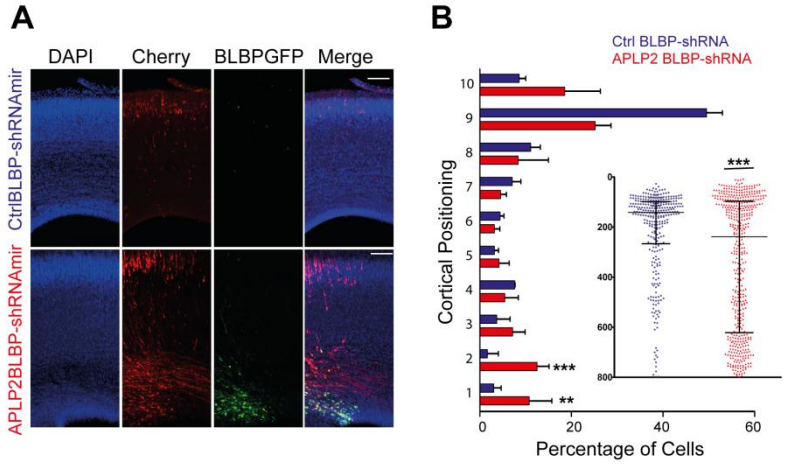

Figure 16. Progenitor specific expression of Aplp2 shRNA is sufficient to retain cells in the proliferative zone of the developing cortex.

A) Confocal images (projection of 10-15 consecutive z-sections) of dko cortical slices transfected with Aplp2 shRNAmir or control shRNAmir under the control of BLBP promoter. Progenitor specific expression of shRNA leads to retention of cells in the VZ/SVZ. Scale bars: 100 µm. B) Quantification of EGFP+ cells of F. Bar graphs represent the frequency distribution of EGFP positive cells in ten equally divided bins from ventricle (1) to the pial surface (10) of the cortical wall. Values represent the mean ± s.d. (n = 3; $^{**}p < 0.01$; $^{***}p < 0.001$, Student's *t*-test). The inset scatter plot compares the population distribution of EGFP+ cells. (n=3, 300-400 cells; Values represent the median ± interquartile range, $^{***}p < 0.001$, Mann Whitney test).

4.7 Cell cycle variables are regulated by Aplp2

In order to understand the role of Aplp2 during precursor proliferation in more detail, we chose to analyse *in utero* electroporated brains two days after electroporation instead of four days. After two days, a substantial number of progenitors can still be found in the proliferative zone of the developing cortex even under control conditions (Tabata and Nakajima 2001; Tabata and Nakajima 2008), allowing us to compare the proliferative fraction of cells under different conditions, whilst after four days most of the progeny deriving from *in utero* electroporated progenitors has been differentiated into neurons (Fig. 7A,B).

Initially, we analysed the position of Aplp2 shRNAmir expressing cells and found a higher number of cells compared to control transfected cells many of which were located closer to the ventricle (Fig. 17A). This initial observation suggested that Aplp2 may be involved in the regulation of progenitor proliferation. Alternatively, the higher number of cells simply

might reflect differences in electroporation efficiencies. To distinguish between these possibilities, we analysed the fraction of EGFP positive cells which were still proliferating under control and “triple ko” conditions. To determine which fraction of the progenitor pool remains in the proliferative state, we examined cell cycle exit rates of EGFP-positive cells after *in utero* electroporation of E14 cortices and BrdU pulse labelling of S-phase cells 24h before collecting the brains at E16.5. This was followed by Ki67 staining, which is expressed throughout the cell cycle and thus labels all proliferating cells (Chenn and Walsh 2002). Hence, the cells which integrate BrdU and express Ki67 after 24h correspond to the proliferative pool, while Ki67 negative but BrdU positive cells correspond to the pool of cells that have recently exited mitosis, and consequently are young neurons (Fig 17B). The rate of cell cycle exit was calculated as the ratio between EGFP+/BrdU+/Ki67- cells and the total EGFP+/BrdU+ population. This ratio was significantly decreased from 74 % in ctrl shRNAmir expressing cells to 45 % in APLP2 shRNAmir dko cells, demonstrating essential functions of Aplp2 in the regulation of neuronal differentiation (Fig. 17B). In light of these findings, the observed decrease in neuronal progression and resulting changes in cortical positioning of “triple ko” cells likely results from delay in the cell cycle exit rate and a delayed entry into the neuronal differentiation program.

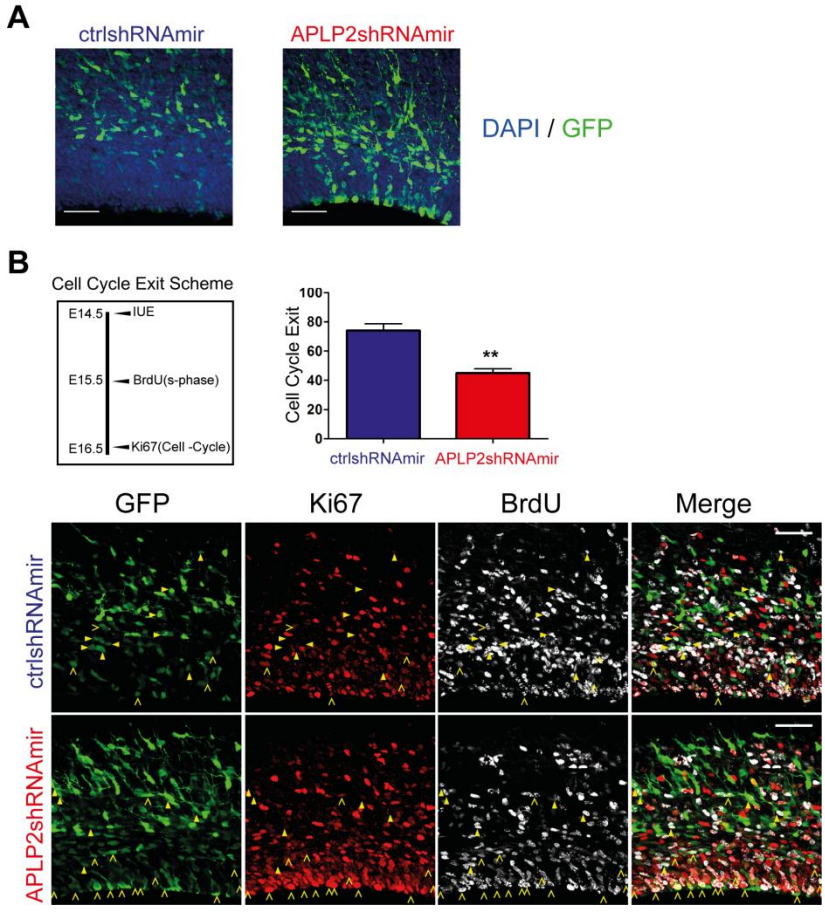

Figure 17. Decreased cell cycle exit rate after Aplp2 down-regulation

A) Confocal images (projection of 10-15 consecutive z-sections) of E16 dko cortical slices co-transfected with Aplp2 shRNAmir or control shRNAmir at E14. Two days after electroporation more cells are found close to the VZ of the developing cortex in Aplp2 shRNA expressing cortices. Scale bars: 50 µm. **B)** Confocal images of dko cortices transfected by Aplp2 or control shRNA at E14.5 followed by BrdU injection at E15.5 and then triple stained for EGFP, BrdU and Ki67 at E16.5 (see scheme of the cell cycle exit assay). The graph shows the decreased cycle exit of Aplp2 shRNA expressing progenitors that is calculated by the ratio of EGFP+/BrdU+/Ki67- cells (filled arrows) divided by the total EGFP+/BrdU+. Open arrows indicate the cells that did not leave the cell cycle. Values represent the mean ± s.d. (n=5; $**p < 0.01$; Student's *t*-test). Scale bar: 100µm.

We reasoned that a reduced cell cycle exit rate can result in more mitotic cells in "triple ko" conditions. To test this hypothesis, we labelled specifically mitotic cells using an antibody against phosphorylated histone 3 (Ser10). Two days after electroporation a two-

fold increase in the number of mitotic cells was observed in VZ/SVZ of dko cortices electroporated with Aplp2 shRNAmir when compared to control shRNAmir (Fig. 18).

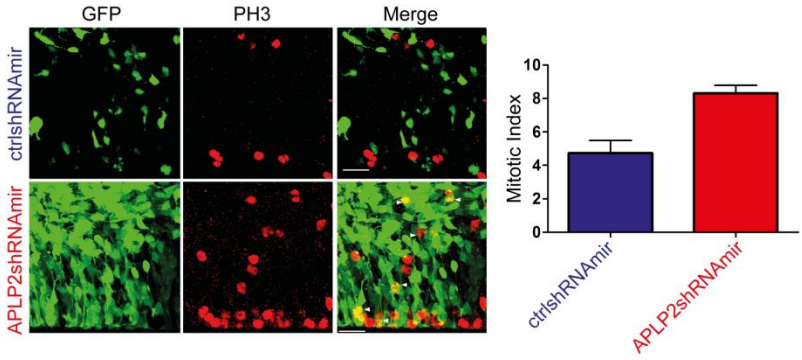

Figure 18. Aplp2 down-regulation increases mitotic index.

Confocal images cells labelled with the mitotic PH3 marker. Two days after Aplp2 down-regulation the number of mitotic cells increased (filled arrows). Graph: mitotic index is calculated by the ratio of PH3+ cells divided by the total number of EGFP+ cells (values represent the mean ± s.d. (n=3; ***p* < *0.01*, Student's *t*-test). Scale bar: 25 µm.

The observed changes in progenitor proliferation raised the question of whether this had resulted in a different distribution of the progenitor and neuronal pools. The balance between progenitors and post-mitotic neurons depends on the ratio of neurogenic *vs.* proliferative radial glia cell division. A proliferative division can be self-renewing or leading to the generation of a radial glia cell and intermediate progenitor cells. These two principle classes of progenitors can be distinguished by specific molecular markers: Sox2 for radial glia and Tbr2 for intermediate progenitor cells. We therefore used double-staining of Sox2 and Tbr2 to evaluate the balance between proliferative and neurogenic division of radial glia cells after Aplp2 down-regulation. Aplp2 down-regulation in dko cortices significantly increased both Sox2 and Tbr2 EGFP-positive cells with a corresponding relative paucity of post-mitotic neurons (Fig. 4D). This result shows that lack of Aplp2 shifts radial glia cells towards proliferative division, implying that Aplp2 is involved in neurogenic division**.**

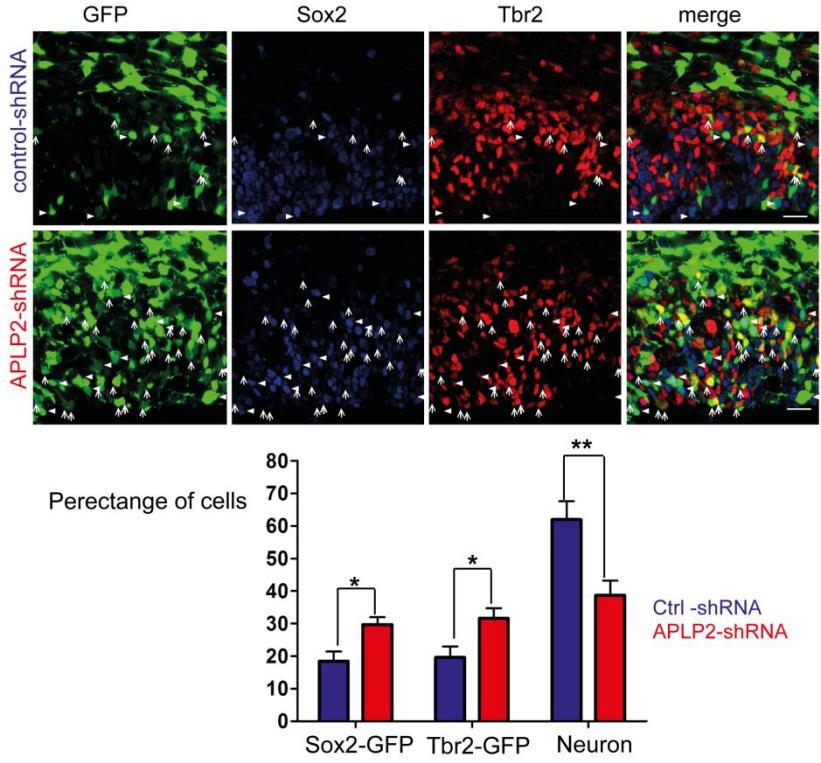

Figure 19. Aplp2 down-regulation increases the proportion of two major classes of cortical progenitors.

Confocal images of Sox2-Tbr2 double stained slices transfected with Aplp2 shRNA or control shRNA. EGFP/Sox2 double positive progenitors: filled arrows; EGFP/Tbr2 double positive progenitors: open arrows. The graph shows that both classes of progenitors are increased after Aplp2 down-regulation (values represent the mean ± s.d. (n=3; **p* <0.05, ***p* < 0.01, Student's *t*-test). Scale bars: 20 µm.

Chapter V: General discussion and perspectives

Our data are consistent with the view that Aplp2 plays a key role in the differentiation of a neuronal progenitor into a neuron. The control of this step is essential during cortical development since there is a close link between the timing of the cell cycle exit and the determination of the laminar fate of the generated neurons (McConnell and Kaznowski 1991). Hence, prospective cortical architecture is already determined at early developmental stages.

We could unravel this function of Aplp2 by combining *in utero* electroporation with cell-specific approaches. In this section, I propose different hypotheses that can explain how Aplp2 can regulate neural stem cell differentiation. Next, I take the example of APP gene family to discuss the advantages and disadvantages of the *in utero* electroporation approach. I end the discussion by explaining the discrepant observations on the different roles for App and Aplp2 in neuronal differentiation and migration and I will speculate on the future direction of research with regard to the physiological function of App proteins during cortical development.

5.1 Hypotheses on Aplp2 regulation of neural stem cell differentiation

We show that reduced levels of Aplp2 in *App/Aplp1* dko progenitors leads to slower cell cycle exit and preservation of progenitors in their proliferative stage (Fig. 19). Aplp2 is a transmembrane protein with a large extracellular multidomain region and an intracellular domain and thus could regulate the proliferation of progenitors functioning either as a receptor, a cell adhesion molecule or a signal transducer. The intracellular YENPTY domain of Aplp2 can bind to several adapter molecules which are involved in the control of neurogenesis such as Dab1 (Homayouni, Rice et al. 1999; Lakomá, Garcia-Alonso et al. 2011), Numb (Roncarati, Šestan et al. 2002) and Fe65 (Ma, Futagawa et al. 2008). Numb could regulate the decision to stop proliferation and promote differentiation as it represses Notch activity (Roncarati, Šestan et al. 2002). Active Notch is promoting the self-renewal of radial glia progenitors (Yoon, Nery et al. 2004), the population which is also increased after Aplp2 down-regulation in progenitors of dko mice(Figs. 3F, 4D). Therefore, one can hypothesize that Aplp2 promotes the neuronal differentiation by directly or indirectly relaxing Notch activity.

Aplp2 might regulate the neural stem cell proliferation by interacting with components of the cell cycle. Interestingly, the conformation and thus binding properties of the Aplp2

YENPTY interaction domain can be influenced by phosphorylation of Thr^{668} by a Cdk1 kinase which couples Aplp2 function and metabolism to the cell cycle (Suzuki, Ando et al. 1997). Similar to Aplp2, Cdk1 expression is also concentrated in the VZ/SVZ of the developing cortex (Visel, Thaller et al. 2004; Diez-Roux, Banfi et al. 2011) suggesting that Cdk1 could be involved in the regulation of neuronal development through Aplp2. Supporting a signalling role for Aplp2 in cell cycle, an analysis of proteins interacting with Aplp2, and not App and Aplp1, revealed members of RhoGTPase such as Rac1 and RhoA (Bai, Markham et al. 2008), which can potentially influence cell cycle progression (Vidaki, Tivodar et al. 2012; Yang, Wang et al. 2012). Moreover, a recent DNA microarray transcriptome profiling of the adult prefrontal cortex showed that the expression of genes involved in neurogenesis is altered in *Aplp2* ko brains (Aydin, Filippov et al. 2011). In this study one interesting candidate was Cdk inhibitor p21 which was down-regulated in *Aplp2* ko mice. p21 down-regulation was found to enhance progenitor proliferation in the adult hippocampus (Pechnick, Zonis et al. 2008). More recently, it was shown that p21 negatively regulates the expression of Sox2, a key regulator of neural stem cell proliferation (Marqués-Torrejón, Porlan et al. 2013). These data can link Aplp2 to components of cell cycle and it will be interesting to determine whether similar mechanisms are relevant for embryonic neurogenesis regulation.

Finally, Aplp2 can influence cell cycle progression by regulating cell-cell and cell-extracellular matrix interactions (Soba, Eggert et al. 2005; Müller and Zheng 2012) could directly regulate the decision between progenitor proliferation and differentiation into neurons, which is strongly dependent on specific membrane associated factors (Temple and Davis 1994). Indeed at the neuromuscular junction, APP family proteins are proposed as novel synaptic adhesion molecules (Wang, Wang et al. 2009) and it will be of interest to investigate a similar role in the context of neuronal progenitor differentiation.

5.2 *In utero* electroporation to study neurodevelopmental processes: the example of APP proteins

Previously, we approached the function of APP protein family by using *in vitro* differentiation of mouse embryonic stem cells. With this approach, we managed to analyse sufficient numbers of App triple ko neurons and neuronal progenitors *in vitro*. We did not find, however, any phenotype in triple ko neurons produced *in vitro*. This prompted us to

further analyse the role of App proteins *in vivo* by generation of chimeric mice using the morula aggregation technique. We succeeded in generating chimeric brains where triple ko neurons were randomly integrated into the wt brain. This technique allowed us to study triple ko neurons in the brain while avoiding lethality of the full triple ko mice. Nevertheless, the yield of the technique was not high. In fact, we obtained only 2 mice with brain chimerism, even though we used more than 1200 morulas in over 30 different experiments (Bergmans, Shariati et al. 2010). In contrast, in this study we used *in utero* electroporation to down-regulate Aplp2 in neurons and neuronal progenitors with a high yield. In our hands, more than 60% of embryos survived the operation and expressed the reporter gene in the brain. Using *in utero* electroporation, we circumvented the lethality of the triple ko mice by down-regulating Aplp2 in a subpopulation of cells in the ventral telencephalon (Fig. 8A). The cells are indeed viable under those conditions as shown by their even increased proliferation rate and their normal morphology (Figs. 10, 11,12). In addition, we performed the experiment in both WT and *App/Aplp1* dko mice which provided us with a system to study "triple ko" neurons and neuronal precursors. Although our studies demonstrate that Aplp2 down-regulation alone is not sufficient to elicit developmental defects (Fig. 7A,B), the expression pattern of Aplp2 suggests a central role in neurogenesis; Aplp2 is specifically distributed in the proliferative VZ and SVZ of the developing cortex. In contrast, Aplp1 is restricted to the CP where differentiated neurons reside while App is distributed in both VZ/SVZ and CP (López-Sánchez, Müller et al. 2005). Thus, App residing in the VZ/SVZ could be responsible for a compensating effect on proliferation in the single Aplp2KO mice.

In utero electroporation is applicable in studies in which the cell autonomous functions of proteins are investigated. This technique allows studying the effect of genetic manipulation of cells in the subpopulation of cells in the wt brains. It is difficult to evaluate exactly the electroporation efficiency but we estimate that in our hand 5-10% of the cells in the targeted region were EGFP positive (after conservative dissection of the targeted region under the dissection microscope). This mosaicism allows to compare transfected cells with the neighbourhood cells that are unaffected and thus to investigate the cell-autonomous function of Aplp2. However, we cannot exclude the possibility of missing some phenotypes that are regulated by soluble fragments of Aplp2. One should also consider the possibility of non-autonomous effects on the cells that are not electroporated.

We could also approach the cell-specific function of App and Aplps by combining *in utero* electroporation with a microRNA based system for shRNA expression. This combination allowed us to narrow down the function of Aplp2 specifically to neural stem cells.

Nevertheless, it should be taken into account that the choice of microRNA is critical for the functionality of shRNA. For example, in our hands the commonly used miR-30 scaffold did not work efficiently in the developing cortex. In fact, it has been shown that down-regulation efficiency by miR-30 varies among different cell types (Liu, Xu et al. 2010). This might be due to the variability in the post-transcriptional processing of precursor transcripts of microRNAs. We chose Let7F scaffold for two reasons: First, it is enriched in the developing cortex; second, it is naturally expressed from an intron. We embedded the Let7F in a synthetic intron to avoid interfering with untranslated regions of the transcripts which are important for translation efficiency and stability of the transcripts.

We used *in utero* electroporation to target the dorso-medial part of the developing cortex. Other groups have targeted other regions of the brains such as the prefrontal cortex or the ganglionic eminence to study tangential migration of inhibitory neurons (LoTurco, Manent et al. 2009). This can be done by changing the position of electrodes. In our experience, however, the precise control of which region is targeted was difficult because movements of the embryos during electroporation. Therefore, a high level of technical proficiency is required to spatially target different regions of the developing brain reproducibly. It is also reported that the long term effect of genetic manipulation by *in utero* electroporation can be studied postnatally. Two technical notes should be mentioned on the application of *in utero* electroporation for long-term analysis of the effect of genetic manipulation. First, the survival of the newborn mice depends on the maternal instinct of the mice which varies from strain to strain. Cross-fostering, gentle handling of the animals and a quiet mice room can improve the survival of the newborn pups. Second, the developmental activity of the DNA construct may decrease during the maturation of the neurons. The choice of promoter is one of the primary factors that need to be considered when designing long-term experiments.

5.3 Controversies on the role of APP proteins in cortical development

To our surprise, we did not observe migration phenotypes in App/Aplp1 dko neurons nor did we detect a migration phenotype in neurons expressing Aplp2 shRNA or "triple ko" neurons. This was consistent with our previous findings using triple ko neurons derived from ES cells (Bergmans, Shariati et al. 2010). In fact, our study had shown that Aplps and App appeared largely dispensable for the radial migration of cortical excitatory neurons. Cortical neuron positioning in *App/Aplp1* double knock-out mice or in APLP2 knock-down

cells was indistinguishable from that in wt mice (Fig. 7A,B). Using *in vitro* assays, we showed that Aplp2 down-regulation does not change migratory behaviour of neurons (Fig. 10). The absence of a migration defect contrasts with other findings showing abnormalities in neuronal migration during cortical development. Yet, the findings of these previous studies were not conclusive, one study showing a complete inhibition of cortical plate entry and the other an ectopic accumulation of neurons in the marginal zone (Herms, Anliker et al. 2004; Young-Pearse, Bai et al. 2007).

However this delay in cortical neuron progression towards the CP is likely a consequence of a primary defect in progenitor function: First, we could not detect any differences in migration speed of isolated "triple ko" neurons and wt neurons using an *in vitro* assay (Fig. 10 A,B); second, we could phenocopy the accumulation of cells close to the VZ/SVZ by expressing Aplp2 shRNA only in progenitors of dko mice (Fig. 16 A,B). Indeed the effect of APP proteins on progenitor function could be a possible alternative or additional explanation for the observed cortical positioning defects in previous publications. This provides an interpretation that is different from what was proposed in the previous papers which considered migration defects as the primary cause. It should be noticed that those previous studies did not directly study migration. Timelapse microscopy could give insight in this particular question. With regard to these discrepancies, one should also consider that acute down-regulation of APP in a wt background may not have the same outcome as a germline deletion of APP which can trigger early developmental adaption.

Taken together, the current data support a model in which App is functional in both progenitors and post-mitotic migrating neurons, whilst Aplp2 is playing a specific role in regulating progenitor proliferation and differentiation.

5.4 Perspective on biological function of APP protein family during cortical development.

The current findings indicate that App and Aplps are essential for correct completion of neurodevelopmental processes such as differentiation, migration, synaptogenesis and circuit formation. One of the first essential decisions for proper formation of the cortex is the exit from the cell cycle and the differentiation into postmitotic neurons. In fact, the prospective identity of a neuron is determined when its progenitor undergoes the last mitosis. The link between Aplp2 and cell cycle during cortical development opens avenues towards the understanding of new functions of App proteins during cortical development. However, the picture remains unclear. One remarkable question remains how Aplp2

signalling is integrated in the global signalling process and how it is responding to external cues that promote cell cycle progression and neuronal differentiation. It is possible that Aplp2 functions as a cell surface receptor responsible for the correct completion of the neurogenic program. Identification of the putative ligand would be possible by using cross-linking and proteomics approaches. Along the same line, *in vitro* culture of neural stem cells is a valuable strategy to test the function of soluble signalling factors that promotes neuronal differentiation after down-regulation of Aplp2. Although this model presupposes a signalling role for Aplp2, one should not neglect the cell adhesion properties of Aplp2. Cell biological studies of Aplp2 subcellular localization and identification of the site of adhesion (apical or basal) are required to understand the link between the Aplp2 adhesion properties and cell cycle progression of cortical progenitors.

It becomes clear that while investigating the biological function of App and Aplps, we lack deeper mechanistic insight. In this regard, defining the molecular interaction network of App and Aplps in different subregions and cell types of the developing cortex is of primary importance. It would be interesting to categorize shared and distinct interactors of App and Aplps. Sometimes APP is dubbed "All Purpose Protein" to indicate the confusing many different signaling pathways and protein interactions in which APP has been implicated. Maybe this information has to be re-evaluated taking into account temporary and cell type expression patterns of APP family and the interacting proteins. It is indeed likely that App and Aplps rewire their interaction network according to the cell type to serve different functions. This will call attention to the cell specific function of App and Aplps. For example, the primary attention has been on understanding the neuronal function of App and Aplps. However, it is now clear that development of the circuits requires the interaction of neurons with each other as well as with glia. Until now, the function of App and Aplps in inhibitory neurons, astrocyte and microglia is not well-studied. Therefore, with the development of the conditional mouse models, it will become feasible to untangle cell specific functions of the APP protein family.

Proposing App and Aplps as cell receptors may lead to a static view on their signaling role without considering their processing and its products. Although, we focused our studies on understanding the cell-autonomous functions of Aplp2, the regulated processing of Aplp2, and App, can mediate non-autonomous functions. This model hypothesizes the existence of a receptor for the soluble fragments of App and Aplps. Therefore, an effort in the field should also focus on the identification of the receptor for App and Aplps and the physiological importance of this interaction.

Finally, generation of specific inhibitor of enzymes involved in amyloidogenic processing of APP such as the BACE1 and γ-Secretase are viable approaches for treatment of Alzheimer's disease. Such compounds would ideally lower the Abeta burden without affecting processing of other substrates. No matter how challenging this might be, changing the metabolism of APP inevitably interferes with the physiological functions of APP raising the possibility of mechanism based side effects. At first glance, developmental functions of APP and APLPs appear a remote link to Alzheimer's disease. However, many neurodevelopmental processes continue to be active in the adult brain in processes such as adult neurogenesis. In fact, generation of neurons continues in two distinct neurogenic niches of the adult brain, i.e. in the subgranular zone of the hippocampus and the subventricular zone of lateral ventricles. Interestingly, hippocampus circuitry that is involved in memory formation is one of the primary regions affected in Alzheimer's disease. Whether and how APP proteins can contribute to generation of neurons in the adult brain and during aging remain unclear. Therefore, a clear understanding of the function of APP will be complementary to the pharmaceutical efforts to treat Alzheimer's disease.

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3. Arefian E, Kiani J, Soleimani M, Shariati SA, Aghaee-Bakhtiari SH, Atashi A, Gheisari Y, Ahmadbeigi N, Banaei-Moghaddam AM, Naderi M, Namvarasl N, Good L, Faridani OR. Analysis of microRNA signatures using size-coded ligation mediated PCR. Nucleic Acids Res, 2011. Citation: 4

4. Ahmadbeigi N, Soleimani M, Gheisari Y, Vasei M, Amanpour S, BagherizadehI, Shariati SA, Azadmanesh K, Amini S, Shafiee A, Arabkari V, Nardi N. Dormant phase and multinuclear cells: two key phenomena in early culture of murine bone marrow mesenchymal stem cells. Stem Cells Dev, 2011. Citation: 6

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6. Shariati S.A, Mehrdad Behmanesh, Hamid Galehdari. Multiplex Tetra-Primer Amplification Refractory Mutation System PCR to genotype SNP8NRG221533 of NRG1 gene. Iranian Journal of Biotechnology, 2008. Citation: 1

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